CARBOHYDRATES IN MARINE ALGAL PROTEINS

by

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TO MY PARENTS

AND ROSEMARY

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I N T R O D U C T I O N

The algae belong to the Thallophyta, a group of plants without differentiation of a shoot into axis and leaf which is a characteristic of the higher forms of plant life. They are divided into four main groups on the basis of colour difference - the Cyanophyta, the Chlorophyta, the Phaeophyta and the Rhodophyta.

The chromoplasts of the red algae, the Rhodophyta, and the chromatophores of the blue-green algae, the Cyanophyta, contain red and blue water-soluble pigments. These were first observed by Von Esenbeck[1] in 1836 and in 1843 Kutzing[2] named the red pigment phycoerythrin and the blue pigment phycocyanin. The pigments have also been found present in a group of marine microflagellates, the cryptomonads[3] - classed as Cryptophyta, an algal phylum.

Rhodymenia palmata of the division Rhodophyta is a member of the Florideophyceal class of the order Rhodymeniales. It is known in Scotland and Ireland as Dulse and is an edible plant found quite commonly in British coastal waters. The name is derived from Rhodon (a rose), humen (a membrane) and palmatus (like the palm of a hand), and this perfectly describes the palm-shaped, red-purple, membranaceous alga which grows on rock surfaces and parasitically on laminaria. This alga is an ideal source of phycoerythrin.

The pigments are always found in chloroplasts or associated photosynthetic lamellar structures - rupture of cells and plastids releases these pigments.

In 1910 Molisch[4] demonstrated that the phycoerythrins and

phycocyanins were proteins and that they could be crystallised from ammonium sulphate solutions. The current designation of the pigments is biliproteins, coined by O'hEocha in 1958. This relates the chromophore of the pigments to a set of chemically similar compounds found in the bile. Biliprotein replaces terms used previously in the literature such as phycochromoproteids, bilichromoproteins or tetrapyrrylproteins.

Early interest in the biliproteins centred on their physiological activity and function in the algae. It was known in the mid 19th century that solar energy utilised by plants was initially absorbed by pigments present in their structure and that the absorption of this energy depended upon the intensity and spectral quality of the incident light. The solar energy used for growth by most plants was found to be that fraction absorbed by chlorophyll "a". This absorption of light and subsequent complex mechanism by which the light energy is used in the fixation of carbon dioxide is known as photosynthesis.

Early work by Engelmann[5] showed that the light absorbed by biliproteins was also available for the process of photosynthesis in algae. Only in deep water are these pigments required to play a major part in energy fixation. Thus Engelmann proposed that the biliproteins provided a means by which light of wavelength 450-530 mμ, i.e. the blue and the green regions, could be absorbed and utilised in the photosynthetic cycle. The 450-530 mμ range is the only band available at great depth of water (50 ft.) and is a region in

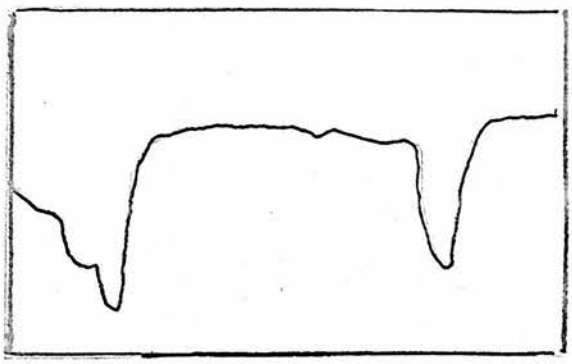


FIGURE 1 A CHLOROPHYLL

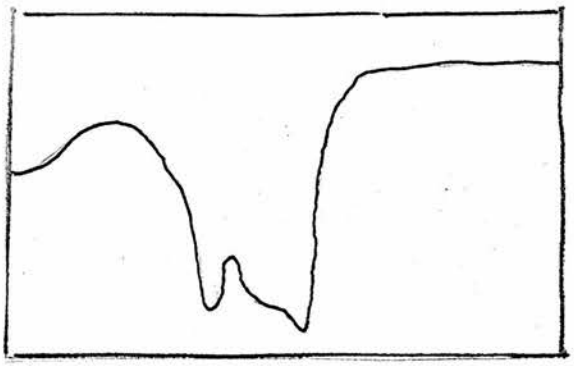


FIGURE 1 B PHYCOERYTHRIN

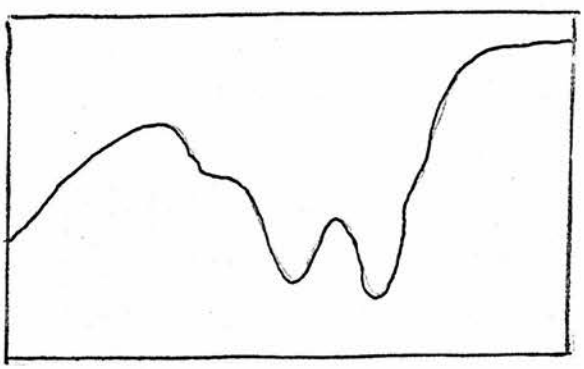


FIGURE 1 C PHYCOCYANIN .

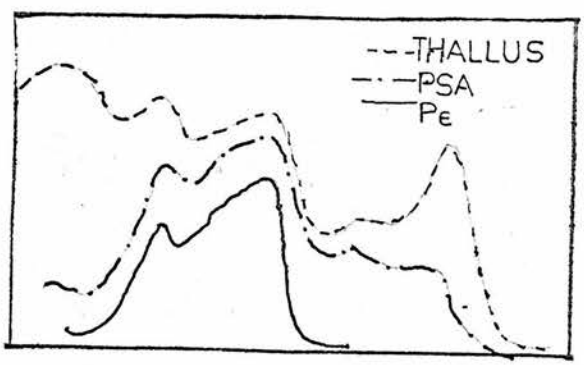


FIGURE 2 ABSORPTION AND PHOTOSYNTHETIC ACTION SPECTRA FOR PORPHYRA NEREOCYSTIS .

SEE SCALE SLIDE IN BACK COVER .

which chlorophyll does not absorb and in which the biliproteins do absorb (Figure 1).

In algae which contain both phycoerythrin and phycocyanin it has been noted that the ratio of the former to the latter increases with the depth of water in which the alga is growing. This can be regarded as a simple proof of Engelmann's theory since phycocyanin cannot absorb in the blue and green regions available at great depth and is therefore of no value photosynthetically. Indeed Kylin[6] has reported a deep growing Rhodophyta containing phycoerythrin only.

Certain blue-green algae are known to contain phycocyanin only, and ~~the~~ a highest concentration of this pigment in red algae is found in green Thalli of Porphyra perforata.[8] A corresponding broadening of the photosynthetic action curve towards the orange-red part of the spectrum (600-650 mμ) demonstrates the photosynthetic effectiveness of phycocyanin.[8]

More recent experiments have revealed that Engelmann's theories are correct and have given further insight into the mechanism of the biliprotein part of the photosynthesis. Dutton,[9] French and Young,[10] and Duysens[11] have shown that light absorbed by the biliproteins and other accessory pigments in algae is able to excite chlorophyll fluorescence. The biliproteins can be regarded as photosensitisers and the process is best described in the form:

$$Pe + h\nu \longrightarrow Pe^{*}$$

$$Pe^{*} + Ch \longrightarrow Ch^{*} + Pe$$

$$Ch^{*} \downarrow$$

$$Ch + E$$

In a typical experiment Haxo and Blinks[8] demonstrated the accessory function of phycoerythrin in Porphyra nereocystis; results can be obtained with other accessory pigments. They compared the photosynthetic action spectra of oxygen evolution (using the polarographic technique for dissolved oxygen) and the absorption spectra of the Thallus and of extracted phycoerythrin (Figure 2).

An unusual feature of this and other experiments is that light directly absorbed by chlorophyll is utilised less effectively than light absorbed by the accessory pigment. One explanation of this has been offered by Duysens who postulated that in red algae two types of chlorophyll were present (the first a synthetically active and fluorescent type and the second non-active and non-fluorescent). The light energy absorbed by the accessory pigment would be transferred to the active chlorophyll "a". This would account for the photosynthetic action spectrum and the chlorophyll "a" spectrum not coinciding.

However, recent studies by Emerson[12,13] have shown that temperature and supplementary lower-wavelength light influence the far-red absorption of algae (i.e. the chlorophyll "a" absorption region). From this Emerson has concluded that total photosynthetic efficiency can be achieved only when light absorption by chlorophyll "a" is associated with the absorption of an accessory pigment. The quanta from the light absorption of chlorophyll "a" must necessarily be accompanied by energetically larger quanta, i.e. quanta available from the accessory

pigment absorption. This effect of supplementary light is known as the Emerson effect or Photosynthetic Enhancement.

As yet no direct evidence has been offered to determine whether the Emerson or the Duysens hypothesis is correct. Whether there are two forms of chlorophyll "a" present or merely one of a low activity which can be enhanced by supplementary absorption cannot be decided on the basis of the available evidence.

Early studies and modern investigations have shown that the absorption of light by a living plant parallels the spectral absorption properties of the extracted pigments and many assumptions have been made from this. However, we must really regard these pigments more as parts of a microscopic organ, the chloroplast, rather than as chemical substances of constant composition which are themselves capable of specific physiological activities.

One of the most characteristic physical properties of biliproteins, which has been mentioned previously in connection with their physiological activity, is the characteristic absorption spectrum exhibited in both the ultra-violet and the visible regions of the light spectrum.

In the ultra-violet region the phycoerythrins and phycocyanins both show absorption maxima at around 275 mμ and 365 mμ. The absorption at 275 mμ has been ascribed to the aromatic amino-acids.[14] Phycoerythrin from most algae also exhibits a third maxima at 305-310 mμ which has been considered a characteristic of the phycoerythrin structure.

TABLE 1

Alga	Author	Max. (mu)	
Porphyra tenera (R)	Fujiwara	615	
	Tiselius	610	552
	Kitsato	610	546
	Svedberg	610	552
Porphyra perforata (R)	Jones & Blinks	615	557
Porphyra pseudolinearis (R)	Takagi	610	552
Ceramium rubrum (R)	Svedberg	621	546
Aphanizomenon flos aquae (C)	Svedberg	615	
Lyngbya lagerheimii (Allo)	Haxo	650	
Porphyra naiadum (C)	Jones & Blinks	615	
Smithora naiadum (C)	O'hEocha	618	
Hemiselmus virescens (E)	O'hEocha	646	583
Arthospira max (Allo)	O'hEocha	654	
Arthospira max (C)	O'hEocha	618	
Porphyra naiadum (Allo)	Jones & Blinks	650	
Porphyra perforata (Allo)	Jones & Blinks	650	
Porphyra nereocystis (R)	Jones & Blinks	620	557
Porphyra nereocystis (Allo)	Jones & Blinks	650	

TABLE 2

Alga	Author	Max. (mu)		
Porphyra tenera (R)	Fujiwara	560	546	496
	Tiselius	560	546	496
	Kitsato	560	526	495
	Svedberg	555	534	495
Porphyra pseudolinearis (R)	Takagi	553	533	495
Prophyra perforata (R)	Haxo	562		497
Porphyra nereocystis (ℛ)	Jones & Blinks	565		495
Porphyra naiadum (β)	Jones & Blinks		545	
Ceramium rubrum (R)	Svedberg	555	529	496
Rhodymenia pacifica (R)	Haxo	564	537	497
Rhodymenia palmata (R)	O'hEocha	568	540	509 (June)
		565	542	502 (Jan.)
Phormidium etocarpi (ℰ)	Haxo	565	542	
Phormidium percinum (C)	Haxo	560		
Smithora naiadum (B)	O'hEocha	560	542	

The letter prefixes, C-, B-, R-, were originally used to indicate the algal source of the biliproteins, i.e. Cyanophyta, Bangiales, Rhodophyta; however, these prefixes are now used as a means of visible spectral classification.

Phycocyanin spectra are less complicated in the visible region than phycoerythrin spectra and can be divided into three types: R-phycocyanin, C-phycocyanin, and allo-phycocyanin, all of which can be differentiated by the positions of their absorption maxima:

R-phycocyanin	550 mμ	615 mμ
C-phycocyanin	615 mμ	
allo-phycocyanin	650 mμ	

Table 1

The general patterns of absorption also vary slightly with the source of protein. Table 1 lists phycocyanins prepared from various algae.

Phycoerythrins also may be characterised on the basis of three absorption maxima at 495 mμ, 540 mμ, and 565 mμ. They exhibit an even greater variability than the phycocyanins, but, like the phycocyanins, they are classified into three main types:

R-phycoerythrin	540 mμ	565 mμ	495 mμ
B-phycoerythrin	540 mμ	565 mμ	
C-phycoerythrin	565 mμ		~~495 mμ~~

Table 2

The phycoerythrins show differences in the extinctions at absorption maxima as well as differences in the wavelength at these maxima. In some R-phycoerythrins, for example, the maximum at 540 mμ is missing or is replaced by a shoulder.

The spectra of the biliproteins have also been found to vary seasonally[15] and have on occasion been found to vary with the means of preparation.[16,17]

Kylin[18] and Kitsato[19] were the first workers to show an interest in the prosthetic groups of biliproteins. They subjected the proteins to enzymatic and acid hydrolysis but were unsuccessful in obtaining a prosthetic group free from covalently linked polypeptide chains. The prosthetic group of the biliproteins, unlike that of the analogous protein, haemoglobin, is bound tightly to the polypeptide chains of the protein moiety and only drastic reagents cleave the linkage. None of the reagents which have been used to date for removing the chromophore from these proteins are mild enough in action to ensure isolation of the original tetrapyrrole structure and may result in secondary alteration of the prosthetic group.

Lemberg[20] and Siedel[21] were the first workers to relate the chromophores of the biliproteins to the Bile Pigments, and Lemberg named these prosthetic groups phycoerythrobilin and phycocyanobilin to emphasise the relationship. Lemberg described the first successful extraction of a prosthetic group from phycocyanin in which the protein was digested with concentrated hydrochloric acid at 80-85°C for thirty minutes.[20] This chromophore, of molecular formula $C_{34}H_{44}O_8N_4$, constituted 1.5% of the dry weight of the protein. The chemistry of phycocyanobilin is similar to that of various Bile Pigments and by comparison of ultra-violet and visible absorption spectra and responses of the compound to reduction and further chemical

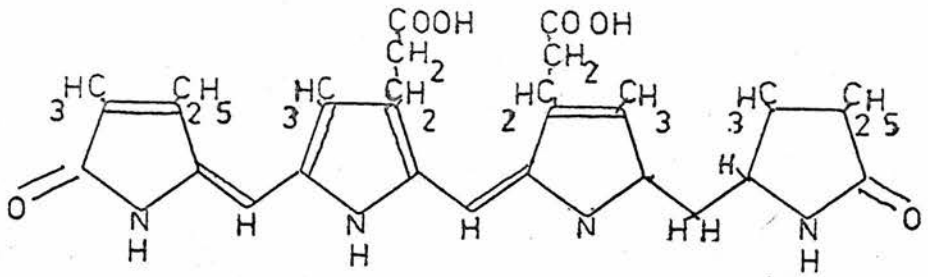
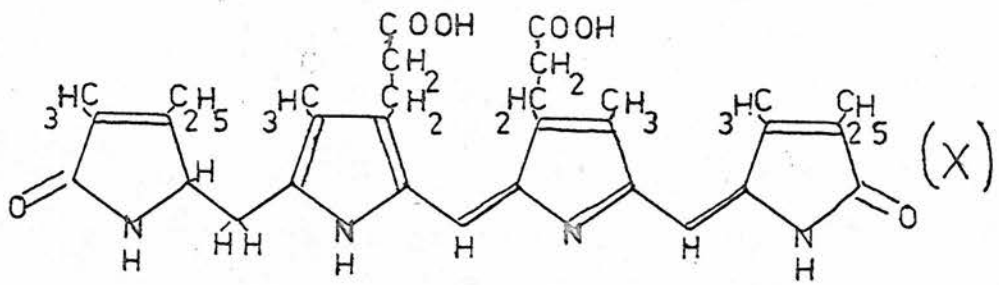


FIGURE 3 LEMBERG PHYCOCYANOBILIN STRUCTURE .



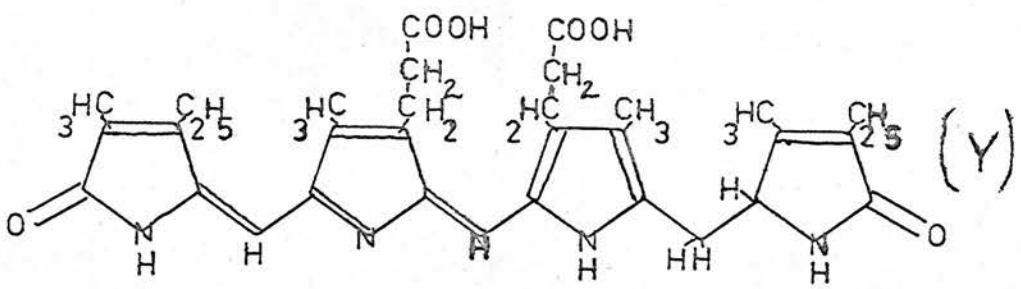


FIGURE 4 (X AND Y) O'HEOCHA STRUCTURES OF PHYCOERYTHROBILIN .

reactions including breakdown of the tetrapyrrole structure to single units, various deductions regarding its structure were made. Lemberg finally assigned to phycocyanobilin a structure of the mesobiliviolin type[22] (Figure 3).

O'hEocha[23] using slightly milder techniques of hydrolysis (12 N HCl at room temperature) was able to isolate a phycobilin from phycocyanin which was not the same as that obtained by Lemberg. It could be converted to the mesobiliviolin-type structure using conditions similar to those used by Lemberg after the original hydrolysis. This indicates that secondary alterations of the prosthetic group must have occurred during the Lemberg extraction. The other possibility that different types of a phycocyanin (i.e. different algal source) have different prosthetic groups has been investigated by O'hEocha.[24] The findings indicate that variation in experimental technique is responsible for variations in the phycocyanobilins rather than existence of more than one phycocyanobilin. No exact structure has yet been ascribed to phycocyanobilin although it is probable that the phycobilin (isolated by acid hydrolysis of phycocyanin) showing an absorption maximum at 630 mμ is the intact chromophore. The light absorption and extinction properties of phycocyanobilin are intermediate between those of the verdins and mesobiliviolin but no fully acceptable structure has as yet been proposed for phycocyanobilin.

The hydrolysis and extraction technique used by Lemberg gave rise to great difficulties, but he managed to isolate the phycoerythrin chromophore as a chloroform-soluble methyl ester.

The chemistry and absorption spectrum of this phycoerythrobilin closely resembled that of phycocyanobilin and thereby established that it too was a tetrapyrrole structure of the same type as the bile pigments.

O'hEocha[25] obtained a chloroform-soluble phycoerythrobilin using a milder hydrolysis technique than Lemberg. This phycoerythrobilin was characterised by absorption maxima at 312 mμ and 576 mμ in acid chloroform or 593 mμ in neutral chloroform (cf. phycocyanobilin 630 mμ). The chromophore was strongly attached to the protein and also extremely labile; this has made elucidation of its structure exceedingly difficult.

Recent studies[25] have shown that this phycobilin extract is probably the chemically unaltered prosthetic group; and that the "phycoerythrobilin" isolated by Lemberg was a urobilin artefact of the native prosthetic group. The structure of phycoerythrobilin was described as $\underline{\underline{X}}$ or $\underline{\underline{Y}}$ by O'hEocha (Figure 4). The Lemberg pigment, whose structure was assigned by Siedel[26] and was previously recognised in the literature as phycoerythrobilin has been renamed mesobilirhodin.

The prosthetic group has been calculated by O'Carra to account for 7.2% of the weight of phycoerythrin from absorption spectral data and this agrees more closely with further protein analyses figures than the previous analytical data for the prosthetic group obtained by Lemberg. We can calculate from O'Carra's data and the known molecular weights of the chromophore (ca. 600) and the protein (290,000) that one molecule of phycoerythrin must have 35 phycoerythrobilin prosthetic groups.

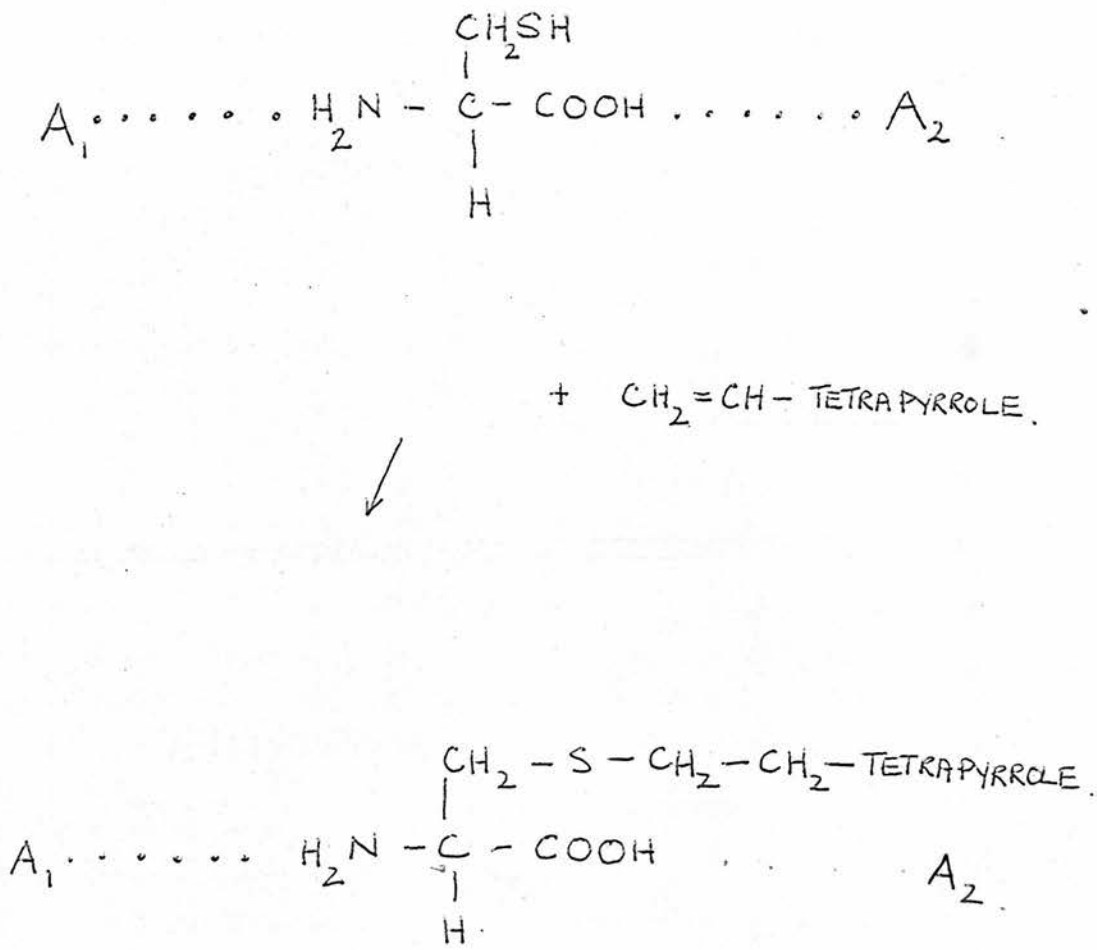


FIGURE 5 PROPOSED STRUCTURE OF [redacted] PROTEIN LINKAGE. WITH MONOVINYL UROBILIN FROM PHYCOERYTHROBILIN.

This suggests that the protein is an aggregate of subunits. The subunit of M.W. 17,000 reported by Vaughan (Ph.D. Thesis) would have two attached chromophores on this basis.

The results from investigations of hydrolysis of the chromophore suggest that the linkages between the chromophore and the polypeptide moieties must be very stable. The exact chemical nature of this linkage has as yet not been established although several investigators have advanced theories. Lemberg[27] suggested that the linkage might involve a peptide bond between the free acidic groups on the propionic acid side chains on the 4 and 5 positions of the tetrapyrrole and free amino groups, in, say, a lysine residue. However, recent investigations[28] have shown that all the ϵ-amino groups and all the N-Terminal amino groups were free to react with dinitrofluorobenzene. As these are the two most likely positions for the chromophore to be attached by such a peptide link, it seems that this linkage is unlikely. The rate of hydrolysis of the chromophore, which appears to be too large for a peptide bond, makes the theory even less plausible.

O'Carra and O'hEocha[25] have shown that an extracted phycoerythrobilin will react with various compounds containing the thiol group, e.g. ethyl mercaptan. They propose that the phycoerythrobilin undergoes a prototropic isomerisation to a monovinylurobilin which is able to react with a thiol grouping to give a stable linkage (Figure 5). Attempts to cleave this bond using the Paul reagent applied so successfully to cytochrome-c[29] have so far failed. To account for this it has

been suggested that strong interactions exist between the protein moiety and the chromophore. Such interactions have been shown to exist by O'Carra.[430] who compared the spectra of phycoerythrin and its phycobilin, and phycocyanin and its phycobilin, in neutral solutions. They stated that the only explanation possible for such large spectral differences as occur between a biliprotein and its phycobilin is some kind of interaction between the chromophore and the protein, as the protein moiety alone does not contain groupings which could give rise to the observed additional absorptions in the visible region.

A survey of phycoerythrins and phycocyanins from various sources shows that although the intact proteins vary spectrally, the spectra of their respective phycobilins are identical. These spectral differences can, therefore, only be explained in terms of interactions between the chromophore and the protein moiety. Jones and Fujimori[30] also provided evidence to support a theory that sulphydryl and disulphide bonds are in some way involved in the interaction. These authors showed that heat denaturation and peptic digestion removed these interactions and suggested that this indicated a similarity between the interactions in the phycobilins and those in cytochrome-c.[29] The intense fluorescence characteristic of biliproteins is reversibly quenched in acid solution but extreme changes of pH result in change of the colour and intensity of fluorescence. Lemberg concluded from these facts that the acid, in quenching fluorescence, unmasked the pyrrole

nitrogens; and in this unmasked form these were shown to be able to complex with zinc. Since native biliproteins are metal free, Lemberg reasoned that the fluorescence in native phycobilins must be dependent on a labile linkage between the pyrrole nitrogens and the peripheral hydrogen atoms of the protein moiety. O'hEocha provided further evidence for this secondary type of bonding with experiments using the hydrogen bond-cleaving reagent, 8M urea.

In conclusion, while there has been no definite assignment of the chromophore-protein linkage in phycoerythrin, several worthwhile theories have been proposed, none of which can be completely discounted, and any combination of which is possible.

The Molecular Weights of the biliproteins were investigated during the period 1920-1940 when Svedberg and his co-workers applied ultracentrifugation techniques to phycoerythrins and phycocyanins from a variety of algal sources. In 1928 Svedberg and Lewis[31] determined by means of Sedimentation Equilibrium and Sedimentation Velocity that the phycoerythrin of Ceramium rubrum had a M.W. of 208,000 ± 8,000 and the phycocyanin a M.W. of 106,000 ± 5,000. In 1929, Svedberg and Katsurai[32] compared the Molecular Weights of the biliproteins in Porphyra tenera and Ceramium rubrum and found that the respective M.W.s were identical. However, an investigation of the phycocyanin from Aphanizomenon flos aquae showed that the biliprotein was a mixture of molecules of M.W. = a, 3a, 6a where a = 34,600. They also showed that the M.W.s were pH-dependent.

Svedberg and Eriksson[33] in 1932 investigated the M.W.s of biliproteins from various algal species and showed that ammonium sulphate precipitation had no effect on the M.W. They pursued earlier work indicating that M.W. was pH-dependent and showed that while phycocyanin was stable between pH = 2 and pH = 5, it disaggregated at pH = 5.8 to form molecules of half the original size. Eriksson-Quensel[34] continued her work on pH stability and published results of studies on phycoerythrin in 1938. She determined the M.W. of phycoerythrin to be 290,000 with an improved technique; and this figure is now generally accepted. She found that the molecule was stable between pH = 3 and pH = 10 and that it dissociated at pH = 11.4. More recently Krasnovskii[35] investigated phycoerythrin from Callithamnion ribosum and his figures agree with the currently accepted M.W. value of 290,000-300,000. The results of Airth and Blinks[36] studies on some plant proteins also agreed with this figure.

We can conclude that the Molecular Weight of R-phycoerythrin is in the range 290,000-300,000 and the Molecular Weight of R-phycocyanin is 270,000 irrespective of the algal source. However, these data only apply to the R- types of biliprotein - the B- and C- types apparently differ physically as well as chemically from the typical Rhodophycean biliproteins.

Although the polypeptide chains constitute over 80% by weight of the biliprotein molecules, little is known about the structure of this protein moiety. Most of the published work

TABLE 3 (Ref. 37)

Amino acid composition of phycobilin chromoproteins isolated from three species of Porphyra

(Percentage by weight)

Amino acid	P. naiadum				P. perforata			P. nereocystis	
	PC	PE	APC	"I"	PC	PE	APC	PC	PE
Aspartic	12.9	15.3	12.8	12.9	10.7	12.5	9.4	12.6	11.3
Glutamic	12.3	13.9	16.3	17.5	12.9	10.1	11.2	12.5	8.7
Serine	3.1	0.2	3.5	3.1	2.4	2.5	3.5	8.9	8.0
Glycine	5.4	5.7	7.2	5.2	6.8	9.1	9.2	11.9	13.5
Threonine	5.8	1.6	8.6	3.7	6.1	3.6	4.5	5.7	2.8
Alanine	12.3	18.3	10.9	9.5	12.6	20.3	12.5	12.3	11.7
Histidine	1.0	2.1	+	0.8	1.4	2.1	1.0	1.3	1.9
Lysine	2.6	1.4	5.1	2.3	3.1	4.9	3.6	3.4	6.5
Arginine	2.0	1.1	5.8	1.4	2.0	4.6	4.2	2.9	7.1
Proline	5.8	4.1	6.2	4.9	5.1	2.9	5.2	3.8	4.6
Valine + methionine	10.6	7.4	11.7	6.0	10.5	9.1	6.9	6.3	3.1
Phenylalanine	6.5	10.7	3.1	6.3	2.0	4.2	7.4	3.4	5.6
Leucine	11.2	10.1	5.1	10.5	12.2	8.9	11.1	7.8	6.8
Isoleucine	9.1	6.3	3.9	8.8	6.8	4.4	7.8	3.1	5.2
Tyrosine	-	2.0	+	5.0	4.4	1.9	+	4.1	3.7
Cystine	-	+	-	+	-	+	-	-	+

PC = phycocyanin; PE = phycoerythrin; APC = allophycocyanin; "I" = highly ionized fraction. + = present but too small to determine. - = not detected.

TABLE 4 (Ref. 28)

Amino acid	R-PE Rhodymenia palmata	C-PE Phormidium persicinum	R-PE Porphyra tenera	B-PE Porphyridium cruentum
Aspartic acid	10.7	12.5	10.4	11.1
Glutamic acid	9.4	10.3	4.6	8.9
Serine	7.1	8.0	6.6	7.9
Threonine	5.1	3.5	2.6	5.8
Glycine	5.5	8.6	6.8	10.0
Alanine	11.3	15.3	16.3	11.9
Valine	9.9	9.0	11.8	7.1
Isoleucine	4.1	5.0	4.8	4.9
Leucine	11.1	8.5	9.0	7.2
Phenylalanine	4.0	3.2	2.6	4.0
Tyrosine	4.1	2.0	2.0	3.7
Proline	5.4	2.3	4.5	4.3
Histidine	1.7	1.8	3.9	2.0
Lysine	3.9	4.5	2.9	6.2
Arginine	3.7	4.6	7.8	7.0
Methionine	2.2	1.0	2.6	1.0
Cystine	0.8	0.5	0.8	0.5

TABLE 5 (Ref. 38)

Amino Acid Composition of Phycoerythrin

Amino acid	Amino acid in g./100 g. of protein	Amino acid residue in g./100 g. of protein	N in % of protein N	Amino acid in moles/10^5 g. protein
Aspartic acid	10.00	8.65	6.87	75.1
Glutamic acid	4.16	3.65	2.58	28.2
Serine	6.62	5.48	5.75	62.7
Threonine	2.84	2.41	2.19	23.8
Glycine	2.73	2.07	3.34	36.7
Alanine	12.50	9.97	12.80	140.0
Valine	5.80	4.91	4.55	49.5
Isoleucine	3.57	3.08	2.50	27.2
Leucine	5.66	4.87	3.96	43.1
Phenylalanine	1.62	1.44	0.90	9.9
Tyrosine	2.30	2.07	1.16	12.7
Proline	3.86	3.26	3.08	33.5
Histidine	0.70	0.62	1.24	4.5
Lysine	3.88	3.40	5.82	26.6
Arginine	6.03	5.41	12.70	34.8
Methionine	1.74	1.53	1.07	11.7
½ Cystine	0.85	0.73	0.65 (4.59)*	7.1
NH_3	1.88		10.18	
(Amide NH_3)**	(1.73)		(9.35)	
Totals	77.75	63.55	81.35	627.1

* Calculated cystine value deduced methionin from total sulphur values.

** Amide NH_3 was determinated by ninhydrin technique of Hanafusa and Okada.

TABLE 6 (Ref. 39)

Protein:	Phycocyanin-C		Phycoerythrin-R	
Source:	P. tenera		P. tenera	
Molecular weight:	300,000		290,000	
Total N:	18.22		15.59	
Amino acid	1	2	1	2
Glycine	4.38	76.9	3.54	62.1
Alanine	9.70	136.5	10.13	142.6
Serine	7.45	115.7	6.41	73.7
Threonine	4.81	47.8	3.52	34.9
Proline	3.46	35.7	2.23	23.0
Hydroxyproline	0	0	0	0
Valine	6.66	67.3	6.89	69.6
Isoleucine	5.42	47.9	4.17	36.9
Leucine	10.51	93.0	7.05	62.4
Phenylalanine	2.86	19.4	2.27	15.4
Tyrosine	7.45	45.7	4.84	29.7
Tryptophan	nd	nd	nd	nd
Cystine/2	1.47	14.4	2.63	25.8
Cysteine	nd	nd	nd	nd
Methionine	3.66	27.9	2.47	18.9
Aspartic acid	10.83	94.1	11.46	99.6
Glutamic acid	11.71	90.8	7.85	60.9
Amide N	1.40	100	1.35	96.5
Arginine	7.98	51.1	7.78	49.9
Histidine	0.36	2.6	0.95	6.8
Lysine	3.51	27.5	4.54	35.5
Hemin				
Totals	102.2	994.3	88.76	847.7
	96.0		102.5	
Reference no.	119		119	
Hydrolysis/correction	1, 3/Z		1, 3/Z	

TABLE 7 (Ref. 40)

Amino acid	Amino acid residue (g./100 g. of protein)		Nitrogen (% of total recovered nitrogen)	
	R-phyco-erythrin	C-phyco-cyanin	R-phyco-erythrin	C-phyco-cyanin
Asp	10.38	10.82	9.75	9.90
Thr	4.47	5.09	4.79	5.30
Ser	6.75	5.16	8.38	6.23
Glu	6.73	10.54	5.64	8.60
Pro	2.11	2.71	2.35	2.93
Gly	3.72	4.06	7.04	7.48
Ala	8.86	7.86	13.48	12.68
Val	6.31	4.23	6.88	4.49
Met	1.63	0.84	1.35	0.74
Ile	4.44	4.44	4.24	4.13
Leu	7.39	7.69	7.06	7.14
Tyr	4.49	5.28	2.98	3.40
Phe	2.74	3.13	2.02	2.23
Lys	3.68	4.02	6.21	6.60
His	0.59	1.05	1.35	2.42
NH_3	0.70*	-	-	-
Arg	6.73	7.66	13.82	15.27
$CySO_3H$	3.64	0.63	2.61	0.45
Total	84.66	85.21	99.95	99.99

* This value is not included in the total.

has merely involved qualitative and quantitative examination of the amino-acids present. It is now generally agreed that phycoerythrin and phycocyanin contain the normal amino acids found in plant proteins and that the few unidentified amino-acids reported by some of the earlier authors can be regarded as peptides which resulted from incomplete hydrolysis of the proteins. Jones and Blinks[37] separated the amino acids by two-dimensional chromatography and estimated their percentage composition by spot density (Table 3). Raftery and O'hEocha[28] used the two-dimensional chromatographic method of Levy and Chung to separate the amino-acids and estimated them by ninhydrin colorimetry (Table 4). These results can only be regarded as tentative since the advent of ion exchangers has produced more accurate means of quantitative analysis.

Fujiwara[38] used the Moore and Stein technique to investigate the amino-acid composition of the biliproteins found in Porphyra tenera. She found that seventeen common amino acids were present in the relative proportions shown in Table 5. Kimmel and Smith[39] used a modification of the Moore and Stein ion exchange procedure, and reported that 88% by weight and 100% of the nitrogen of these proteins can be accounted for in terms of amino-acids (Table 6). In 1964 Raftery and O'hEocha[40] investigated the amino-acid composition of specially purified phycoerythrin and phycocyanin using an automatic amino-acid analyser. 85% of the total weight of both proteins was recovered as amino-acids (Table 7). These results can be regarded as the most accurate to date. Berns et al.[41] have

investigated the amino-acid composition of the biliproteins with similar results.

The results from the various amino-acid analyses given in the previous section show considerable variation. Although in all cases the same eighteen amino acids have been found, different investigators report differences in amino-acid ratios. The differences even occur in the same variant, R-phycoerythrin from the same source - Porphyra tenera as investigated by Fujiwara, Kimmel and Smith, and Raftery and O'hEocha. Although this situation is not satisfactory, the differences may be a result of the means of preparation and extraction or a result of seasonal changes. The visible spectrum of phycoerythrin has been observed to vary slightly with season and it seems reasonable to suppose a like variation in the amino-acid composition.

All the biliproteins examined have certain common features in their amino-acid composition. They contain large amounts of acidic amino-acids: R-phycoerythrin ca. 8% Glutamic acid and 10% Aspartic acid. This is a most unusual feature since for most plant proteins the reverse is true. All the biliproteins contain large amounts (ca. 9%) of alanine and leucine. The terminal amino-acids have been of interest to several authors. Raftery and O'hEocha[28] used the Sanger and Ednam methods and found that the N-terminal amino-acids in R-phycoerythrin were leucine and serine (10 residues and 9 residues/molecule respectively). Hydrazinolysis results indicated that alanine was C-terminal in R-phycoerythrin (the same acid is

C-terminal in R-phycocyanin). In an investigation of a chromopeptide from R-phycoerythrin, Fujiwara[42] obtained tentative evidence that some of the N-terminal residue may be prosthetic group. She managed to isolate a D.N.P. derivative which was chromatographically similar to D.N.P. bilirubin.

Investigations by O'hEocha of the N-terminal amino acids R-, B- and C-phycoerythrins by the Sanger method indicated that in all cases the N-terminal amino-acid was methionine. Recent work by O'Carra[43] has agreed with O'hEocha's later results and methionine has been agreed upon as the only N-terminal amino acid in the phycoerythrins; R- and B-phycoerythrins were shown to contain 14 methionine residues per molecule and C-phycoerythrin, 8 per molecule. Raftery and O'hEocha[40] recently determined that the C-terminal amino-acid in R-phycoerythrin is alanine and the C-terminal in C-phycocyanin is serine.

Calculations of the isoelectric point of phycoerythrin by Kimmel and Smith,[39] who worked out the resultant pH in terms of acidic and basic amino-acids and amide nitrogen, have failed to find agreement with the experimental value. Two possible explanations for this are that some of the amide groups are involved in further bonding or that a non-amino-acid component is present in the native protein. Both effects could lead to a lower isoelectric point.

Fujiwara attempted to obtain a core chromopeptide from phycoerythrin and discovered that some of the peptides she examined contained bound carbohydrate.[44] After subjecting the protein to hydrolysis with pepsin, trypsin, and Bacillus

subtilis protease, she was able to isolate peptides which contained between 4% and 13% carbohydrate. These results led Fujiwara to investigate the carbohydrate content of the native phycoerythrin.[45] She subjected the undegraded protein to acid hydrolysis and isolated acidic and basic components using DOWEX 50 ion exchanger. The sugar components of the acid fraction were investigated and assayed by the Somogyi, Orcinol, Bial and Carbazole methods. Orcinol and Carbazole results indicated the presence of 3-4% carbohydrate and the reducing value was quoted as 0.5%-1.5% depending on the time of hydrolysis.

The native protein was also methanolised and the resulting methanol fraction of methyl glycosides investigated. Chromatography of the sugars obtained by methanolysis indicated the presence of galactose, xylose, arabinose, two components which moved more slowly than galactose in a basic solvent and one which ran between arabinose and rhamnose. Attempts were made to characterise these minor components. The faster moving one appeared from its reaction with various sprays and its electrophoretic mobility to be rhamnose. One of the slower moving components appeared to be a galactose-containing disaccharide. Amino-sugars were observed in the basic fraction but were not identified. Part of the methanolysate was analysed for 3,6-anhydro-galactose by paper chromatography - a faint yellow spot with o-aminophenol was observed but not enough sugar was available for confirmation by polarography.

Tsuchiya and Sasaki[46] investigated the carbohydrate components of phycoerythrin and phycocyanin extracted from

fresh Porphyra tenera. Investigation after hydrolysis showed that both biliproteins contained carbohydrate. The sugars were tentatively identified as xylose, mannose, glucose and galactose. Phycoerythrin contains a further two sugars and phycocyanin a further three sugars which were not identified.

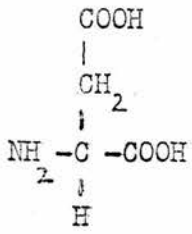
It appears, therefore, that the biliproteins are glycoproteins with a low carbohydrate content and that the carbohydrate content consists of a combination of at least six different sugars. The most recent and most accurate of the amino-acid analyses of phycoerythrin by Raftery and O'hEocha indicates that the amino acids make up 84.7% of the weight of the protein. Fujiwara accounted for a further 4.8% of the protein weight as carbohydrate. O'Carra estimates spectroscopically that the prosthetic groups make up 7.2% of the weight. These analyses figures account for 97.8% of the phycoerythrin molecule.

Many proteins in animal tissue are associated with a prosthetic group which is distinct from the main polypeptide component. The structure of the plant protein, phycoerythrin, is even more complex than this. The results of Fujiwara and Tsuchiya indicate that this protein has two different types of prosthetic groups covalently linked to the polypeptide backbone, namely, tetrapyrrole chromophore and carbohydrate.

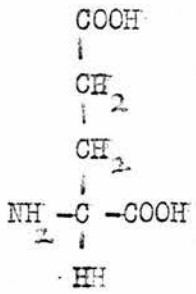
The terms mucopolysaccharide and glycoprotein were for some time regarded as being synonymous and were defined as complex macromolecules which contained as integral parts carbohydrate and amino acids.[47] More recently "mucopoly-

saccharide" has been used to describe polysaccharides involving amino sugars and "glycoprotein", a complex of carbohydrate and amino acids in which the carbohydrate has a relatively low degree of polymerisation and is linked covalently to the polypeptide chain.[47] Phycoerythrin falls into this category and is therefore a glycoprotein as well as a chromoprotein. The isolation, purification, and structural investigation of glycoproteins has proceeded slowly and with great difficulty and until very recently the techniques applied to the structural investigation of glycoproteins were the established methods of classical protein and polysaccharide chemistry which have produced only a very rough picture of the structure of glycoproteins. Indeed, most of the earlier studies were confined to the carbohydrate moieties wherever possible. However, more recent studies have tended towards investigation of the mode of linkage between the carbohydrate and the protein in the complex macromolecule.

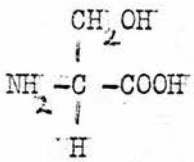
Investigation of a glycoprotein is directed along lines to provide the answers to three main questions. The first of these is: which amino acids and sugars combine to make up the molecule? This question is usually answered by hydrolysing the protein and identifying the subunits by chromatographic techniques. The second question concerns the nature of the carbohydrate in the glycoprotein. Is it in one large unit attached to the protein through one linkage or in a series of smaller units attached at various points to the polypeptide chains? The third problem is that of the mode of linkage.



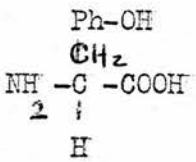
L-ASPARTIC ACID



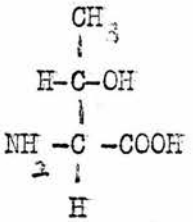
L-GLUTAMIC ACID



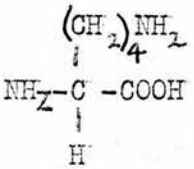
L -SERINE



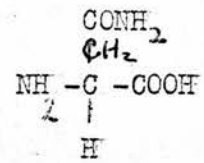
L-TYROSINE



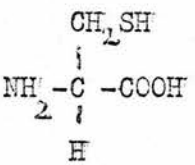
L-THREONINE



L-LYSINE



L-ASPARAGINE



L-CYSTEINE

FIGURE 6 AMINO ACIDS CAPABLE OF FURTHER BONDING .

This is most easily solved by a consideration of protein structures. A protein can be defined as a polymer of amino acids bound together via peptide links. The only possible points of attachment of prosthetic groups apart from the free carboxyl and amino groups at the ends of the chains are the side chains of those amino-acid constituents which have suitable functional groups available for bonding, i.e. groups which are not involved in peptide linkages. These further functional groups found among the common amino acids are the carboxyl group of the dicarboxylic acids, aspartic and glutamic, the amino group in lysine, the amide in asparagine, the hydroxyl groups in serine, threonine and tyrosine and the free thiol group in cysteine (Figure 6).

The sugar units may vary in complexity from the simple pentoses and hexoses through acetylated amino sugars to complicated longer chain sugars such as sialic and neuraminic acids, all of which are normally bound to proteins through their reducing ends.

The chemistry of the submaxillary gland glyooproteins (which are the most powerful of the influenza inhibitors) has been intensively studied. Ovine submaxillary mucin (O.S.M.) has been found to contain equimolar amounts of N-acetyl galactosamine and N-acetyl neuraminic acid. These sugars constitute 42% of the weight of the molecule (a further 52% being amino acid) and are released from the glycoproteins on hydrolysis with barium hydroxide. The carbohydrate structure has been established as

α-D-acetyl neuraminyl-(2 $\rightarrow$6)-N-acetyl-D-galactosamine[48]

and it has been calculated that eight hundred prosthetic groups are distributed along the polypeptide chain. Early work indicated that 80% of these groups were bound by a glycosidic-ester linkage to the free carboxyl groups of aspartyl and glutamyl side chains.[49] More recent investigations have shown that this is not the case and reduction with sodium borohydride and palladium chloride of the base-catalysed cleavage products has given clear evidence that the linkage is a glycosidic bond to seryl and threonyl side chains.[50] Bovine submaxillary mucin (B.S.M.) contains similar prosthetic groups to those found in ovine mucin and recent investigations have shown that they too are linked to the polypeptide chain through serine and threonine residues.[51]

Ovalbumin, a crystalline protein from egg white, is a glycoprotein (M.W. 45,000) containing a low proportion of carbohydrate and contains only one prosthetic group,[52] an oligosaccharide consisting of 5 mannose residues, 3 glucose residues, and 3 acetyl groups. Enzymatic digestion of this protein gave a single glycopeptide which contained all the carbohydrate present in the molecule. The amino-acid sequence in this peptide was shown to be

Try - Asp - (Leu, Ser, Thr) - Val.

\

Carb.

The oligosaccharide was found to be linked to the protein through one specific aspartic acid residue in the protein.

This carbohydrate-amino acid linkage has been studied most intensively and it was established that a glucosamine residue is linked to aspartic acid.[53] Comparisons of electrophoretic behaviour and infra-red and ultra-violet spectra of the glycopeptide and of model compounds indicated that the linkage is between the β-carboxyl group of aspartic acid and the 1-amino group of 2-deoxy-2-acetamido = glucosylamine.[54,55] The structure of the carbohydrate portion of this molecule has not yet been clarified.

Ovomucoid is a glycoprotein secreted by the epithelium of the oviducts of the hen which has a molecular weight of 29,000, 29% of which is carbohydrate. Glucose, galactose and N-acetyl glucosamine were isolated from the acid hydrolysates of this protein. The molecular weights of the glycopeptides obtained by the pronase digestion of the protein were investigated and it was shown that the carbohydrate is not present as a single prosthetic group but as several smaller units separately attached to the polypeptide chain.[56] There are indications that there are three of these units per molecule and that they are linked to the protein moiety through asparaginyl residues,[57] as in ovalbumin.

One of the best characterised of the plasma proteins is the α_1-acid glycoprotein (orosomucoid) which is easily prepared in a state of high purity. The carbohydrate fraction, which varies between 20% and 40% according to species, consists of galactose, mannose, fucose, N-acetyl glucosamine and sialic acid. Studies on this covalently

bound carbohydrate have indicated that it is arranged in five to seven branched polysaccharide units.[58,59] Investigations of homogeneous glycopeptides obtained by proteolsis support this theory. The oligosaccharide units are terminated by:

O-N-acetyl neuraminidyl-(2 → 4)-O-D galactosyl
(1 → 4)-N-acetyl glucosamine[60]

and their core is made up of a sequence of alternating galactose, mannose and N-acetyl glucosamine units. The carbohydrate-protein bond is thought to be

β-aspartyl-N-acetyl glucosaminyl,

again as in ovalbumin.[61]

A further group of serum glycoproteins, the α_2-glycoproteins, which can be resolved into two subfractions rendered insoluble by zinc and barium ions respectively, are very similar chemically to the α_1-glycoproteins.[62] They contain the same sugars and their polypeptide backbones are also comparable. Fetuin, the predominant protein of foetal calf serum, is another similar glycoprotein containing branched oligosaccharides constructed of the same five basic units and all terminated by sialic acid.[63] The Smith technique of serial periodate oxidation involving oxidation followed by reduction and mild hydrolysis has indicated that the oligosaccharides sequences are:

N-acetyl neuraminyl-(2 → 3)- -D galactopyranosyl
-(1 → 4)-N-acetyl glucosamine

and that these chains are attached to an internal core of mannose and N-acetyl hexosamine residues. It is likely that

mannose serves as branching points of these carbohydrate units and the C_1 of N-acetyl glucosamine is involved in the glycopeptide bond.[63,64]

Various blood group-specific substances can be isolated from human ovarian cyst fluid and it has been found that the division of human erythrocytes into four seriological groups can be associated with a relatively small part of the whole specific macromolecule. It appears that most of the activity is associated with certain structural features of the carbohydrate moiety of a glycopeptide isolated from the secretions. These glycopeptides are 80% carbohydrate, which is assembled from L-fucose, D-galactose, N-acetyl-galactosamine, and N-acetyl-glucosamine units, combined with eleven or twelve amino acids and the various group-active substances are generally similar in character with the exception of the features responsible for group-specific activity. Results of partial hydrolysis and hydrazinolysis coupled with the results of immunological and enzymatic techniques have revealed that group-specific activity is associated with terminal carbohydrate units in the oligosaccharide side chains.[65,66,67]

Some rabbit,[68] pig[69] and human[70] globulins have all been proved to be glycoproteins. Combinations of galactose, mannose, fucose, sialic acid and glucosamine are covalently bound to polypeptides of several common amino acids. The carbohydrates are combined in oligosaccharide chains although proteolytic hydrolyses have shown that there must be some three to five points of attachment between carbohydrate and amino

acid. The evidence available to date suggests that the carbohydrate seems to be exclusively bound to aspartic acid residues.

The pituitary gonadotropins, Interstitial-Cell Stimulating Hormone (I.C.S.H.) and Follicle Stimulating Hormone (F.S.H.), and chorionic and thyrotropic gonadotropins have long been recognised as glycoproteins.[71] Recent investigations have demonstrated conclusively that sialic acid is an essential component of all gonadotropins and that its enzymic removal inactivates the hormone. These sialic acid residues occupy a terminal position linked α-ketosidically to the next chain member. Galactose, mannose, glucosamine and galactosamine have all been isolated from gonadotropin hydrolysates.[72] As yet no information regarding carbohydrate-protein covalent linkage has been published, although in I.C.S.H.[73] and also in thyrotropic preparations[74] it has been established that the carbohydrate is located in one prosthetic group attached to the polypeptide through one amino acid residue.

One further animal glycoprotein of interest has been extracted from the tunics of carp bladders, Icthyocol. It has been proposed on the basis of periodate oxidation that the slight percentage of carbohydrate present (1.4 residues glucose and 2.1 residues of galactose per 1,000 amino acids) is attached to the protein through a glycosidic bond and that the remaining 2, 3 and 4 positions of the hexoses are unsubstituted.[75] Recent evidence has been presented which indicates that this hexose in icthyocol and in collagens in general is firmly bound to the protein and probably takes part

in the cross-linking of the polypeptide chains.[76]

A rigid, insoluble glycoprotein structure, probably highly polymeric and possibly also cross-linked, is an important constituent of bacterial cell walls. It seems that this rigid structure consists of a backbone of heteropolysaccharide cross-linked by small peptides, and a small molecular weight glycopeptide of this type has been isolated from the dialyzable fraction of lysozyme-digested walls of Micrococcus lysodeikticus.[77] The peptide side chains are joined to the carboxyl groups of N-acetylmuramyl units.

Two very complicated glycolipids which are really peptide-gluco-lipids have been isolated from Bacterium avium. They both have the partial formulae

R-CO-D-Phe-D allo-Thr-D-Ala-D allo-Thr-D-Ala←6 deoxy L-talose,

3-OMe-6 deoxy L-talose

3,4-di-O-methyl-L-rhamnose

and it has been established that whereas some of the sugar moiety is bound to threonine in what is a familiar glycopeptide bond, other sugar units are ester-linked to the C-terminal alanine residue.[78]

Although several plant and bacterial glycoproteins have been investigated, glycoproteins from plant sources have been neglected and at this time only a few tentative results concerning plant glycoproteins are available.

Barley albumin is one of the few plant glycoproteins to have been investigated.[79] Hochstrasser managed to isolate a

basic, reducing, ninhydrin-positive substance from a formic acid hydrolysate of barley albumin treated with trypsin. This substance when isolated and subjected to acid and alkaline hydrolysis, methanolysis, methylation, dinitrophenylation and osazone formation proved to be 4-L-alanyl xylopyranose.

The coagulable and thermostable fractions of extracted barley albumin were submitted to enzymatic hydrolysis and the hydrolysates could be resolved into six and four peptides respectively.[80] These peptides were shown to contain as integral parts xylose, arabinose, glucose, and glucoronic acid, aspartic and glutamic acids, glycine, alanine, threonine, and serine. Evidence so far obtained indicates that the carbohydrate is bound glycosidically through serine and threonine and as ester-bound carbohydrate to aspartic and glutamic acids. Investigation of certain peptides indicated that aspartic acid is the main component in these glucopeptide bonds.

Neukom and Kundig investigated the glycopeptide fractions of soluble wheat flour pentosans which contain galactose, arabinose, and xylose. They showed that the principal glycopeptide fraction can be cleaved by oxidising agents and a fragment enriched in galactose has been isolated from the products after oxidation.[81]

E X P E R I M E N T A L

GENERAL PROCEDURES

G.P.1. One dimensional descending paper chromatography

Chromatograms were run on Whatman No. 1 paper unless otherwise stated, using control sugars or amino-acids and air drying.

a) Principal Chromatographic Solvents

Ratios expressed as volume to volume.

A. Ethyl acetate; pyridine : water (10; 4:3).

B. Ethyl acetate; acetic acid; formic acid : water (18; 3; 1:4).

C. Butanol; ethanol : water (4; 1:5).

D. Butanol; water : acetic acid (62; 26:12).

b) Detection reagents

The following chromatographic sprays were used to develop chromatograms where appropriate.

I. An 0.5% solution of p-anisidine hydrochloride in butanol was applied to the chromatogram in a fine spray. The chromatogram was then heated at 120°C for three minutes. Chromatograms were observed in normal and ultra-violet light.

II. A 10% aqueous solution of silver nitrate (1 ml.) diluted with acetone (100 ml.) was used as a dipping reagent. The silver nitrate impregnated chromatograms were dried in air (15 min.) and dipped in an alcoholic solution of sodium hydroxide (0.5 M). This latter reagent was prepared by dissolving sodium hydroxide pellets in the minimum amount of water, and making up to the required volume with either ethanol or methanol. The background colour of the chromatograms was

removed by immersion in (1) 0.1 M sodium thiosulphate and (2) water.[82]

III. Ninhydrin. An 0.5% solution of indanetrionehydrate in water-saturated butanol was used as a spray reagent. The chromatograms were heated at 80°C for 5 min. and the spot colour intensified by exposing the chromatogram to a current of steam.

IV. Acetylacetone-reagent.[83]

1. Acetylacetone

A. 0.5 ml. acetylacetone in 50 ml. n-butanol.

B. 5 ml. 50% potassium hydroxide in 20 ml. ethanol.

0.5 ml. of B was added to 10 ml. A.

2. N,N-dimethylaminobenzaldehyde

1 g. reagent in 30 ml. ethanol and 30 ml. concentrated hydrochloric acid. Diluted to 100 ml. with butanol.

The chromatogram was sprayed with (1) and heated to 105°C for 5 min., then sprayed with (2) and heated at 90°C for 5 min.

The following have been used to describe the distances travelled by sugars and amino-acids on paper chromatography:

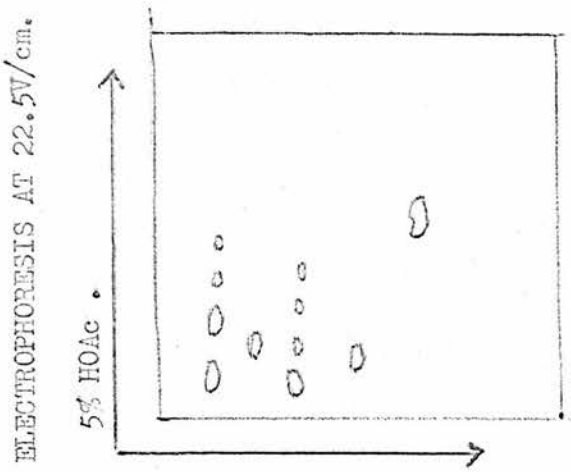
$$Rf = \frac{\text{distance travelled by unknown}}{\text{distance travelled by solvent front}}$$

$$Rgal = \frac{\text{distance travelled by unknown}}{\text{distance travelled by galactose}}$$

G.P.2. Two dimensional paper chromatography

The amino-acid composition of protein and peptide was investigated by two dimensional chromatography.

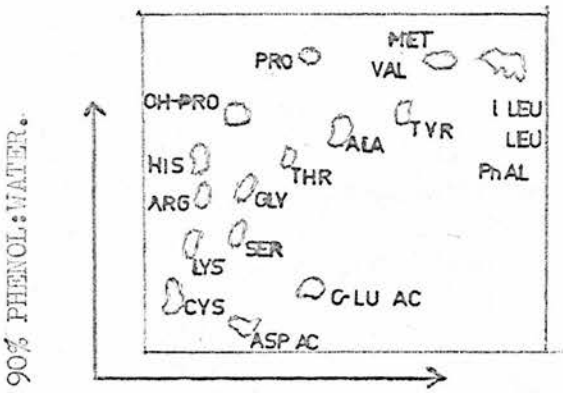
A. Hydrolysates were spotted 12 cm. by 12 cm. from the top



CHROMATOGRAPHY -B.W.A. .

FIGURE 7 TYPICAL PEPTIDE MAP OF A TRYPTIC HYDROLYSATE

ELECTROPHORESIS-CHROMATOGRAPHY .



B.W.A.

FIGURE 8 AMINO ACID MAP . CHROMATOGRAPHY WITH B.W.A. AND 90%PHENOL:WATER .

left hand corner of a 45 cm. by 45 cm. sheet of Whatman No. 1 paper. The chromatogram was run in the first direction with solvent D, dried in air, retrimmed and run at right angles to the previous run in 90% phenol/water. The chromatogram was allowed to dry in air (12 hrs.), washed in ether, and finally allowed to dry for a further 12 hrs. before development with ninhydrin.

A typical amino-acid map as obtained by this method is shown opposite (Figure 7).

B. Similar results were obtained using the same solvents and general techniques applied to ascending chromatography. Chromatograms 20 cm. by 20 cm. were developed four at a time on a chromatogram rack.

G.P.3. Paper Electrophoresis

The apparatus used was of the Wieland-Fischer type. The paper strip which had been previously moistened with buffer was supported between the electrodes by a polythene plate. Spots of samples (amino-acids and peptides) were applied to the moistened paper at points previously marked in pencil.

The constat was capable of voltages up to 450 v at 10-20 ma. without severe overheating.

Separation was best effected on Whatman No. 54 paper at a voltage gradient of 20-22 v/cm.

The chromatograms were developed with ninhydrin.

Principal buffer solutions used were:

A. Pyridine; acetic acid : water (1; 10:289).

B. Acetic acid : water (5:95).

G.P.4. Paper Electrophoresis-chromatography

Peptides from protein were investigated by a mapping technique involving electrophoresis in one dimension followed by ascending chromatography in the other. Samples were spotted 6 cm. by 6 cm. from the corner of a 22 cm. by 22 cm. sheet of Whatman No. 5 paper. After electrophoresis at 450 v in 5% acetic acid for 1 hr. (22.5 v/cm.) the electrophoretogram was dried in air for 3-4 hrs. then chromatographed ascending in Solvent D.

A typical map of a tryptic hydrolysate is shown opposite (Figure 8).

G.P.5. Thin Layer Chromatography

Qualitative investigations of amino-acids, peptides and carbohydrates were carried out using various forms of thin layer chromatography. In this technique a glass plate was covered with an aqueous slurry of absorbent powder so that a uniform coherent film adhered to the glass. After drying and activating the plate, the sample to be investigated was spotted on the film and the charged plate developed in an enclosed tank by an ascending solvent.[84]

Plates used were 5 cm. by 20 cm. (x), 20 cm. by 20 cm. (y), and 2 cm. by 7 cm. (z). Thin films of MN Silica Gel G-HR (Macherey Nagel and Co.), MN Cellulose Powder 300 (Macherey Nagel and Co.), and Keiselguhr G (Merck) were applied to the plates.

The 20 cm. by 20 cm. plates were spread using the Desaga

Spreader, the 5 cm. by 20 cm. using the Shandon Spreading Apparatus and the 2½ cm. by 7½ cm. by hand.

T.L.C.1

Separation of mono- and di-saccharides on Kieselguhr. Ethylacetate; n-propanal : water (39; 14:7) solvent.[85]

T.L.C.2

Separation of monosaccharides on cellulose. Pyridine; ethyl acetate : water (6; 4:3) solvent.[86]

T.L.C.3

Separation of mono- and di-saccharide glucoses on Silica Gel. Butanol; acetic acid; ethyl ether : water solvent.[87] (9; 6; 3:1)

T.L.C.4

Separation of amino acids and peptides on Silica Gel. Butanol; water : acetic acid (62; 26:12) solvent.

G.P.6. Thin Layer Electrophoresis[84]

Ionophoretic separation of amino-acids and peptides was done on thin layers of Silica Gels wet with buffer, using a water-cooled Desaga sealed unit and a constat capable of constant voltage up to 450 v at 5-20 m.a.

Voltage gradient: 20-22 v/cm.

Buffers for ionophoresis:

A. Acetic acid; formic acid : water (120; 276:2000).

B. Pyridine; acetic acid : water (1; 10:1989).

G.P.7. Thin Layer Chromatography-ionophoresis

A two dimensional thin layer peptide mapping technique was

used.[88] The sample was spotted 4½ cm. by 4½ cm. from a corner of a 20 cm. by 20 cm. Silica Gel-coated plate which was developed to within 2 cm. of the plate top by an ascending solvent. After drying at 50°C for 2 hrs. the plate was cooled, moistened with a fine spray of buffer, and an electric field applied for exactly an hour in the Desaga apparatus, at right angles to the direction of chromatographic development. Each map was duplicated by reversing the procedure. The sample was spotted on a damp plate and subjected to ionophoresis for one hour. The plate was then dried and the sample developed in the chromatographic solvent in a second direction.

Buffer: Acetic acid; formic acid : water (120; 276:2000).

Ionophoresis: 450 v at 5-20 ma. for one hour; 22.5 v/cm.

Chromatographic solvent: Butanol; acetic acid : water (62; 26:12).

G.P.8. Evaporations

Evaporations were done under reduced pressure using a rotary film evaporator at or below 40°C or in an air current at or below 40°C.

G.P.9. Hydrolysis

a) Small Scale Hydrolysis of Carbohydrates

The sample (1-5 mg.) was heated at 100°C in a sealed glass tube with N-sulphuric acid (1-2 ml.) for 4-6 hrs. The hydrolysate was neutralised with solid barium carbonate, and

filtered; barium ions were removed by passage through Amberlite IR - 120 (H^+) resin; and the deionised solution was concentrated to a syrup.

b) Small Scale Hydrolysis of Proteins and Peptides

The sample (1-5 mg.) was heated at 100°C in a sealed glass tube with 6 N-hydrochloric acid, for 18 to 20 hrs. The hydrolysate was concentrated to a syrup and the residual hydrochloric acid removed by taking up the hydrolysate in water and evaporating to a syrup six times from water and alcohol. Removal of residual chloride salts was achieved by passing through a weak base ion exchange resin (Permutit Deacidite G).

G.P.10. Dialysis

Protein solutions were dialysed in cellophane bags, or Visking tubes, or through Graver Hi-Sep membrane (Graver Water Conditioning Co., 216, West 14th Street, New York, 11, N.Y.), against running tap water or in a stirred beaker of distilled water. Toluene was added to prevent microbial action.

G.P.11. Gas-liquid partition Chromatography

This was done on a "Pye Argon Chromatograph" with argon as the carrier gas. The stationary liquid phase was supported on acid washed celite (80-100 mesh) and consisted of:

(a) Butane-diol succinate ester,

or (b) Polyphenol ether.

The operating temperature was 175°C for column (a) and 200°C for column (b). The retention times, "T", of the methyl ether

methyl glycosides were quoted relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.[89]

G.P.12. Methylation

Disaccharides, monosaccharides and small glycopeptides were methylated using the Kuhn[89] procedure. The sample (1-2 mg.), silver oxide (100 mg.), dimethyl furan (0.1 ml.) and methyl iodide (0.1 ml.) were mixed together in a darkened tube. The stoppered tube was then shaken at 0°C for one hour, then for six hours at room temperature. The resultant gum was diluted with chloroform, filtered, and the precipitate washed with chloroform to give twenty-five mls. of combined filtrate and washes. The chloroform solution was washed with water; the aqueous layer was then back extracted with chloroform; and the washings and solution combined. This chloroform solution was then washed with two 10 ml. portions of 2% sodium cyanide and three 10 ml. portions of water. These washings were rejected. The chloroform layer was dried over sodium sulphate and evaporated to near dryness at room temperature.

G.P.13. Methanolysis

Methanolysis was carried out by refluxing the sugars with dry methanolic hydrogen chloride (3%) (for ca. 6 hrs.). The methanolic hydrogen chloride was prepared by careful addition of acetyl chloride (5 mls.) to methanol (100 mls.). The solution was neutralised with silver carbonate, filtered, silver salts washed thoroughly with dry methanol, and the combined filtrate and washings concentrated to a syrup.

G.P.14. Sugar-estimation

a) The Phenol-sulphuric Acid Method[90]

Aqueous sugar solution (1 ml.) containing 10-100 µg. sugar was pipetted into Eel colorimeter tubes. Phenol solution (5%, 1 ml.) was added to each tube followed by analar sulphuric acid (5 ml.) from a fast delivery pipette, the stream of acid being directed on to the solution surface to obtain a maximum rise in temperature. After 2 hours the optical density of the solution was measured on an Eel colorimeter (green filter OGR1) and at 487 mµ on a Unicam Spectrophotometer S.P.500 against a water reagent blank. Standard graphs of optical density (E) against ug sugar/ml. were constructed before each set of estimations.

b) p-amino Benzoic Acid Method[91]

Para-amino benzoic acid can be used as a colorimetric reagent in the quantitative determination of micro amounts of hexose and pentose. Sugar solution (0.5 ml. containing 10-100 µg sugar) was pipetted into a glass stoppered tube (6" by $\frac{5}{8}$") and mixed with freshly prepared p-amino benzoic acid stock solution (3 ml.), and stock sulphosalicylic acid solution (3 ml.). Both stock solutions were 1.5% solutions of analar chemicals in glacial acetic acid which had been refluxed for two hours and distilled over p-amino benzoic acid. The tube was then heated for one hour in a boiling water bath and was allowed to cool for six hours before measurement of optical density. Standard curves were prepared before each set of determinations. Absorptions at 375 mµ for hexoses and 530 mµ

for pentoses were measured against water blanks using an S.P.500 spectrophotometer.

G.P.15. Resin Hydrolysis[93]

The resin hydrolysis technique used was a modification of that described by Anastassiadis. The principle is that of replacing direct hydrolysis of a protein with aqueous mineral acid by hydrolysis catalysed by the sulphonic acid groups of a sulphonated polystyrene ion exchanger in neutral solution. This use of resin simplified the separation of neutral and basic components to a single stage process. Samples of glycoprotein (1-100 mg.) were mixed with twenty times their weight of DOWEX 50 x 8 resin in the (H^+) form. (The resin was cleaned by repeated washings with 2 N-sodium hydroxide, water and 2 N-hydrochloric acid.) This resin-protein mixture was added to twice its volume of water and was sealed in a Pyrex glass tube. The sealed tube was stirred continuously by end over end rotation in a water bath heated to 100°C for a period of 18/48 hrs. The tube was finally cooled and opened; and the resin and hydrolysate were added to a small column made from the same amount of resin as that used in hydrolysis. The hydrolysate and water washings (2-3 times the volume of the hydrolysate) were combined (water wash). The column was then washed with 2 N hydrochloric acid (acid wash). Chromatographic examination of the fractions indicated that the amino-acid components and chromophore were quantitatively separated from the neutral sugars which appeared exclusively in the water wash.

G.P.16. Calcium Phosphate Chromatography

Calcium triphosphate was prepared by converting freshly prepared brushite ($CaHPO_4.2H_2O$) into hydroxyapatite ($Ca_5(PO_4)_3OH$).

The calcium triphosphate gel was diluted with inactive celite (1 part phosphate to 3 parts celite).

Columns were washed before use with chromic acid, detergent and water. A slurry of the gel was poured into a water filled column and was allowed to settle under gravity. The column was normally supported on a pad of glass wool and the upper surface protected by silver sand. Samples were applied in a solution of sodium chloride (1%) to columns which had been washed with the same solution.[93]

G.P.17. Sephadex Chromatography

Columns which were normally fitted with sintered glass discs were washed before use with chromic acid, detergent and distilled water. A slurry of the gel was poured into a water filled column and was allowed to settle under gravity, while being stirred. The column surfaces were protected by discs of filter paper. Samples were loaded directly to the column surface.[94]

G.P.18. Ninhydrin Solution Estimation[95]

Stock solutions:

A. 20 g. ninhydrin and 3 g. hydrindantin in 750 ml. methyl cellosolve.

B. Buffer solution of 2720 g. sodium acetate in 2 L water, 500 ml. glacial acetic acid, made up to final volume (5 L).

Add 250 ml. A to B.

Procedure:

Ninhydrin solution (1 ml.) added to effluent (2 ml.), shaken, heated to boiling in a water bath for 5 min., ethanol-water (5 ml., 50-50) added as a diluent, and finally read against a blank at 570 mu (Unicam S.P.500).

Ultra violet and visible spectra were recorded on the Perkin-Elmer Model 137 u.v. automatic spectrophotometer or where only absorption at one wavelength was involved the unicam S.P. 500 was used.

EXTRACTION AND FRACTIONATION

The biliproteins are water soluble and can be extracted by repeatedly soaking an alga in cold water. This process may be speeded up by cutting the algae into very small pieces or by grinding it with sand. These processes are normally performed at low temperatures to prevent denaturation of the protein occurring with rupture of the cell. Fresh, undried alga forms the best source of biliproteins although many Japanese workers have been able to isolate reasonable yields of native proteins from "NORI", a commercial preparation of sun-dried Porphyra tenera.

The aqueous extract is usually purified by sieving through celite pads or by high speed centrifugation and the biliproteins are separated from non-protein material by precipitation with ammonium sulphate. Recently a modification has been suggested by Fujiwara[96] who used Rivanol (2-ethoxy-6,9-diamino acridinium lactate) to precipitate the biliproteins and separate them from mucous substances.

In 1910 Kylin[97] reported that he was able to separate phycoerythrin from phycocyanin by careful precipitation with ammonium sulphate. This method, accompanied by the knowledge that the ammonium sulphate precipitated phycocyanin is more soluble than the precipitated phycoerythrin, has been the basis of most of the quantitative extractions and separations since 1910.[32,98]

Tiselius employed electrophoretic methods to separate the

biliproteins.[99] A complete separation was achieved in alkaline solution in the Tiselius U-tube free boundary electrophoresis apparatus.

Albertson and Nyns[100] applied the technique of countercurrent distribution to the biliproteins found in Ceramium rubrum and it was discovered that the proteins could be separated in a dextran polyethylene glycol phase containing small amounts of potassium salts.

Swingle and Tiselius[101] developed a chromatographic method for the separation of the proteins involving adsorption on tricalcium phosphate/celite columns and subsequent desorption of the individual biliproteins by stepwise elution with increasing molarity of phosphate/citrate buffer. This method has been used by several other authors with considerable success.[14,102]

Nultsch[103] devised a further means of separation of the biliproteins by a gel filtration technique. He separated a cold water extract of Phormidium autumnale, containing phycoerythrin, phycocyanin, and allophycocyanin by elution of a sample from a column of G-25 Sephadex with citrate/phosphate buffer.

These latter chromatographic methods do not work well when scaled up for preparative use and may be best regarded as a means of investigation of purity rather than as a quantitative means of separation. The classical fractional precipitation techniques are more useful for larger scale preparations.

The alga Rhodymenia palmata was harvested from a cove at North Berwick on the East coast of Scotland. The first collection (8 litres of fronds) was made at the November low tide. After careful examination and removal of contaminants and foreign species the alga was washed exhaustively with distilled water; it was cooled to 0°C and was macerated at temperatures not exceeding 20°C using a mechanical disintegrator of the Waring Blender type. The pulp was covered with distilled water (0.1% toluene) and was allowed to stand in a darkened room at 3°C for three days in which time most of the chromoprotein content had been leached into the water. The pulp was sieved through fine muslin and the chromoprotein solution was finally filtered through a celite pad. Extraction was considered complete after five immersions over a period of twenty-two days:

A. 5l for 3 days
B. 5l for 3 days
C. 3l for 3 days
D. 3l for 5 days
E. 3l for 8 days

The filtered extracts were combined and molar calcium acetate was added to the solution until it was 0.1 M with respect to calcium acetate. The extract was then centrifuged using a Sharples Supersonic Bolt Centrifuge and the green, slimy precipitate was discarded. The protein material was then precipitated with ammonium sulphate (400 g/l.), the precipitate centrifuged and then redissolved in water (5 l.) and

dialysed against running tap water at 3°C in the dark for three days. The precipitation with ammonium sulphate, centrifugation and dialysis procedure was repeated and the resulting solution of phycoerythrin and phycocyanin was precipitated fractionally with rivanol (as per Fujiwara[96]). After several preliminary experiments on a small scale it was found that the rivanol precipitation was time-dependent and that to achieve fractionation addition of rivanol had to be immediately followed by centrifugation before mass protein precipitation took place.

The protein solution (5 l.) was thoroughly mixed with a solution of rivanol (12.5 ml. of a 1% aqueous solution). The solution was centrifuged immediately (1,8000 r.p.m. for 20 mins. at 0°C) and the precipitate was discarded.

After a further addition of rivanol (12.5 ml. of 1%) the protein solution was centrifuged (1,8000 r.p.m. for 30 min. at 0°C) and the resultant rivanol symplex was washed with a solution of sodium dihydrogen phosphate (0.5 M, 500 ml. x 3). The precipitate was dissolved in a solution of ammonium sulphate (10%) Fraction A . A third addition of rivanol was made and the solution centrifuged for a further period (30 min. at 1,800 r.p.m. at 0°C). The precipitate was washed with sodium dihydrogen phosphate solution and was dissolved in a solution of ammonium sulphate Fraction B .

The centrifugate and phosphate washings were combined and were stirred with kaolin acid clay (300 g. for 1 hour) to remove rivanol. The solution was filtered and the kaolin, stained with brown coloured material, was discarded. The

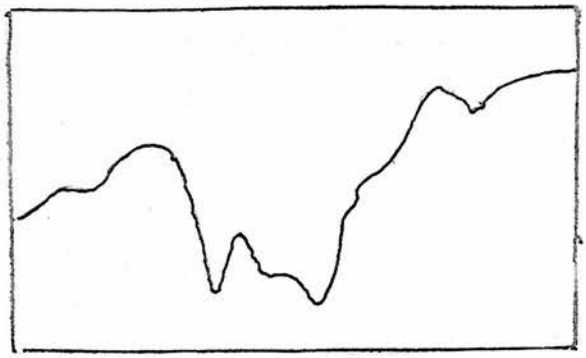


FIGURE9 COLD WATER EXTRACT OF RHODYMENIA PALMATA.

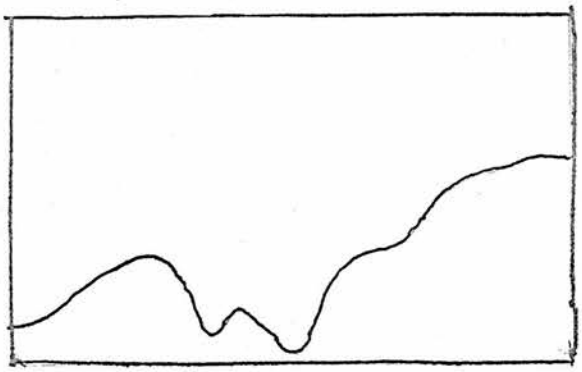


FIGURE10 AMMONIUM SULPHATE PRECIPATE OF COLD WATER EXTRACT

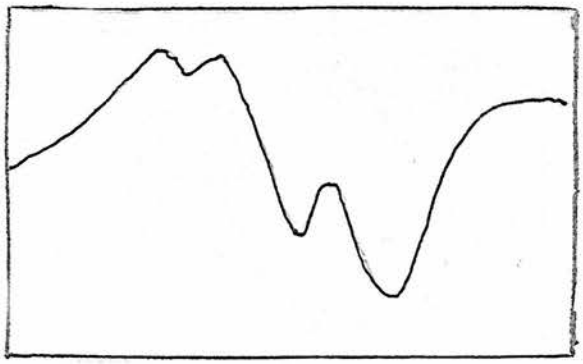


FIGURE11 PHYCOCYANIN EXTRACT II .

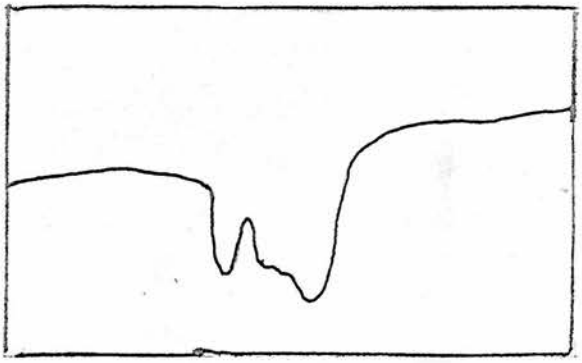


FIGURE12 EXTRACTION I FRACTION A .

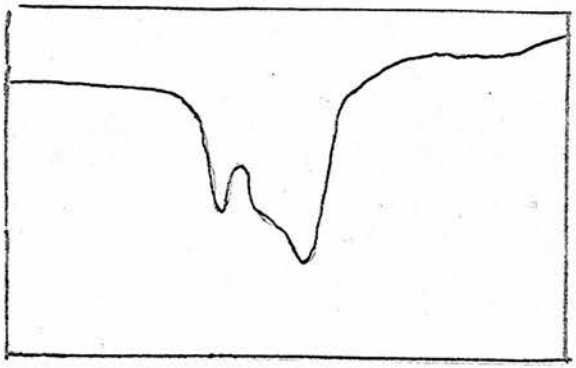


FIGURE13 FRACTION A AFTER $CaPO_4$/CELITE CHROMATOGRAPHY .

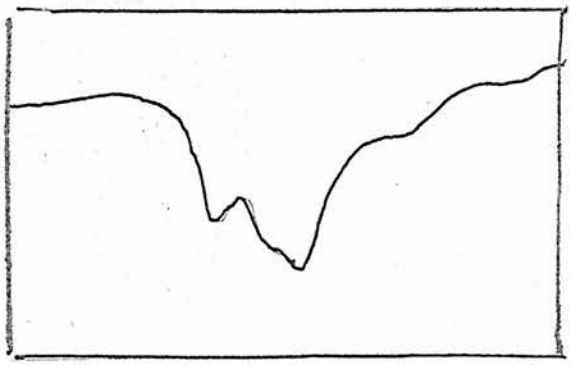


FIGURE14 EXTRACTION I FRACTION B .

purple-blue solution was centrifuged (1,800 r.p.m. for 1 hour at 0°C) and the pH adjusted to six with acetic acid (0.1 M). Ammonium sulphate (200 g/l.) was added and the resultant blue precipitate was centrifuged and dissolved in ammonium sulphate solution (10%) Fraction C .

Each stage of the extraction and fractionation procedure was followed by investigation of the visible region spectrum of each extract and fraction. From the spectral data obtained the number of components present and an approximate estimation of the purity could be deduced. Figure 9 shows the visible spectrum of the water soluble extract of Rhodymenia palmata. The general pattern indicates the major component is phycoerythrin (λ max.: 497 mμ, 565 mμ) and the minor absorption maxima indicate the presence of chlorophyll a (λ max.: 415 mμ, 675 mμ) and that of phycocyanin (overtone at 550 mμ, shoulder at 615 mμ).

After the extract had been centrifuged (Sharples), precipitated (ammonium sulphate) and dialysed, the spectrum (Figure 10) indicated that the extract was now an exclusive mixture of biliproteins and that chlorophyll A had been removed.

The rivanol-precipitated Fraction C was redissolved in distilled water and dialysed against running tap water. The protein solution was pure blue in colour under artificial light and showed the red fluorescence in daylight which is characteristic of R-phycocyanin. The visible spectrum showed that it was almost pure phycocyanin (λ max.: 550 mμ, 615 mμ) (Figure 11) with a trace quantity of phycoerythrin. Several

attempts to crystallise this phycocyanin from solutions varying in ammonium sulphate concentration failed. The protein solution was dialysed till free from salt and was freeze dried. The protein was stored in stoppered vials sealed in polythene bags with Silica Gel as a dessicant at 0°C.

A sample of Fraction A was dialysed against running tap water for two days (0°C). The solution was pure red in colour under artificial light and exhibited a strong orange fluorescence in daylight. The visible spectrum indicated that the fraction was R-phycoerythrin, completely free from phycocyanin (Figure 12). There was an indication from a slight staining of the dialysis membrane that the sample was contaminated with rivanol.

A sample of Fraction A (20 mg.) was chromatographed on a column of calcium phosphate/celite (G.P.16). The sample was introduced to the column (16 cm. by 3 cm.), previously washed with a solution of sodium chloride (1%), by direct loading and was eluted under pressure with a series of McIlvaine buffers (phosphate/citrate buffer pH = 6.5) of increasing molarity. The sample was eluted as a single band at one buffer concentration (0.1 M) leaving only rivanol contamination on the column. The coloured eluate proved to be spectrally identical to Fraction A (Figure 13).

From the spectral and chromatographic evidence it was assumed that Fraction A consisted of phycoerythrin free from the other biliproteins although slightly contaminated with precipitant rivanol. The fraction was dialysed exhaustively

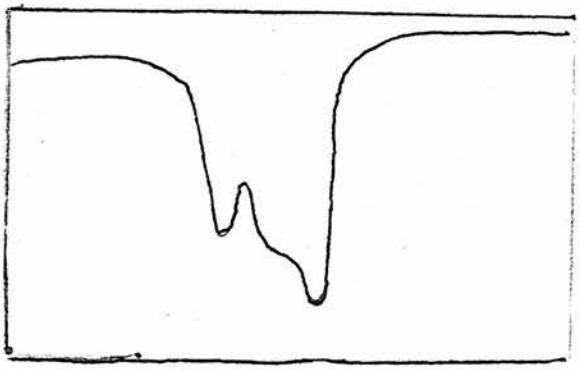


FIGURE 15 PHYCOERYTHRIN B AFTER CaPO /CELITE CHROMATOGRAPHY

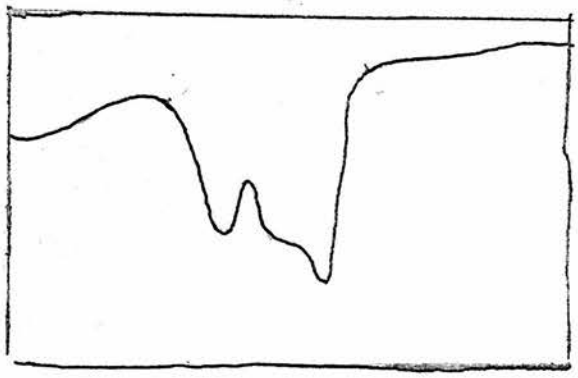


FIGURE 16 FRACTION D (AND E)

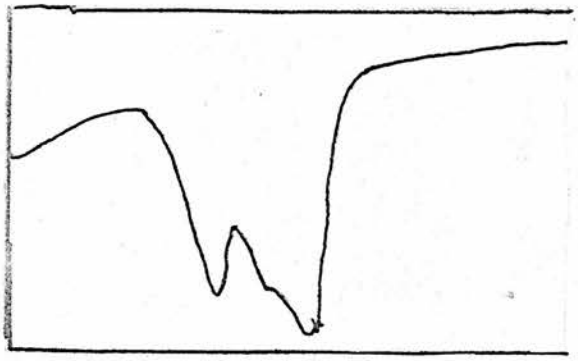


FIGURE 17 PHYCOERYTHRIN EXTRACTION II .

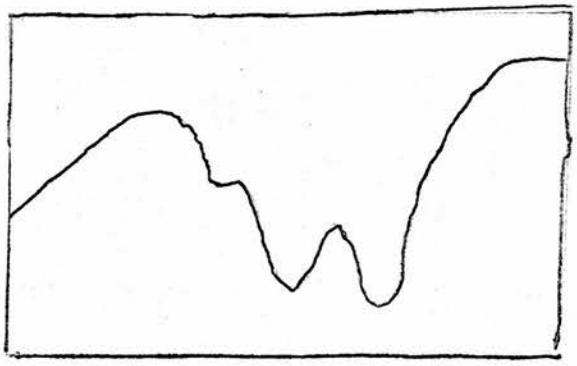


FIGURE 18 PHYCOCYANIN EXTRACTION II .

(0°C) (G.P.10) and was freeze dried before storage in stoppered vials as described previously.

The ammonium sulphate solution of Fraction B was dialysed exhaustively against running tap water (4 days at 0°C). The resultant solution was purple in colour, exhibiting no fluorescence in daylight, and the dialysis membrane was badly stained with rivanol. The ultra-violet spectrum of the fraction revealed that it was a mixture of phycoerythrin and phycocyanin (λmax.: 497 mμ, 565 mμ, overtone 550 mμ, shoulder 615 mμ) (Figure 14). A sample (20 mg.) of the fraction was chromatographed on a calcium phosphate/celite column (G.P.16). The procedure followed was exactly the same as that described for Fraction A. A band of the sample was eluted with McIlvaine buffer (pH = 6.5, 0.01 M) which proved to be pure phycoerythrin (vis. absorption, λ max. 479 mμ, 565 mμ) (Figure 15) and a second band was eluted with more concentrated buffer which was phycocyanin (vis. absorption, λmax. 550 mμ, 615 mμ).

Fraction B was dissolved in sufficient water to give a final volume of 3 L and the solution was cooled to 0°C. A solution of rivanol (1%, 6 ml./L) was mixed with the protein solution and centrifuged (1,800 r.p.m., 30 mins. at 0°C). The resultant precipitate was washed with a solution of sodium dihydrogen phosphate (0.5 M, 200 ml. x 3) and was dissolved in a solution of ammonium sulphate (10%) (Fraction D). The rivanol centrifugate and the phosphate washings were combined and were stirred with kaolin (100 g. for 1 hr.). The solution was filtered and centrifuged, and its pH adjusted to six with acetic

acid. Ammonium sulphate was added (200 g./L) and the resultant precipitate was centrifuged and dissolved in ammonium sulphate (10%) (Fraction E). Chromatographic and spectral examination (Figure 16) indicated that Fractions D and E were respectively phycoerythrin and phycocyanin which were completely separated but grossly contaminated with rivanol which quenched their natural fluorescence. A series of ammonium sulphate precipitations followed by dialysis was to some extent effective for removal of rivanol but at the same time resulted in loss through denaturation and it was felt advisable that some other method of rivanol removal be found. The fractions were finally dialysed exhaustively against running water ($0^{o}C$) and were freeze-dried and stored in this freeze-dried state. It could be concluded that although rivanol precipitation provided a means for separation of phycoerythrin and phycocyanin it had the disadvantage that the removal of the last traces of contaminating rivanol was difficult. Rivanol contamination was easily detected by its inhibition of the natural orange fluorescence of phycoerythrin. A solution of rivanol-contaminated phycoerythrin was purple in colour in contrast to the red, orange-fluroescing native phycoerythrin.

A standard sample of phycoerythrin to be used for comparisons was prepared. A sample of Fraction B (50 mg.) was chromatographed on calcium phosphate/celite prepared in the usual way and the band eluted with 0.01 M McIlvaine Buffer (pH = 6.5) was dialysed and freeze-dried. This freeze-dried sample was redissolved with McIlvaine Buffer (20 ml., 0.1 M)

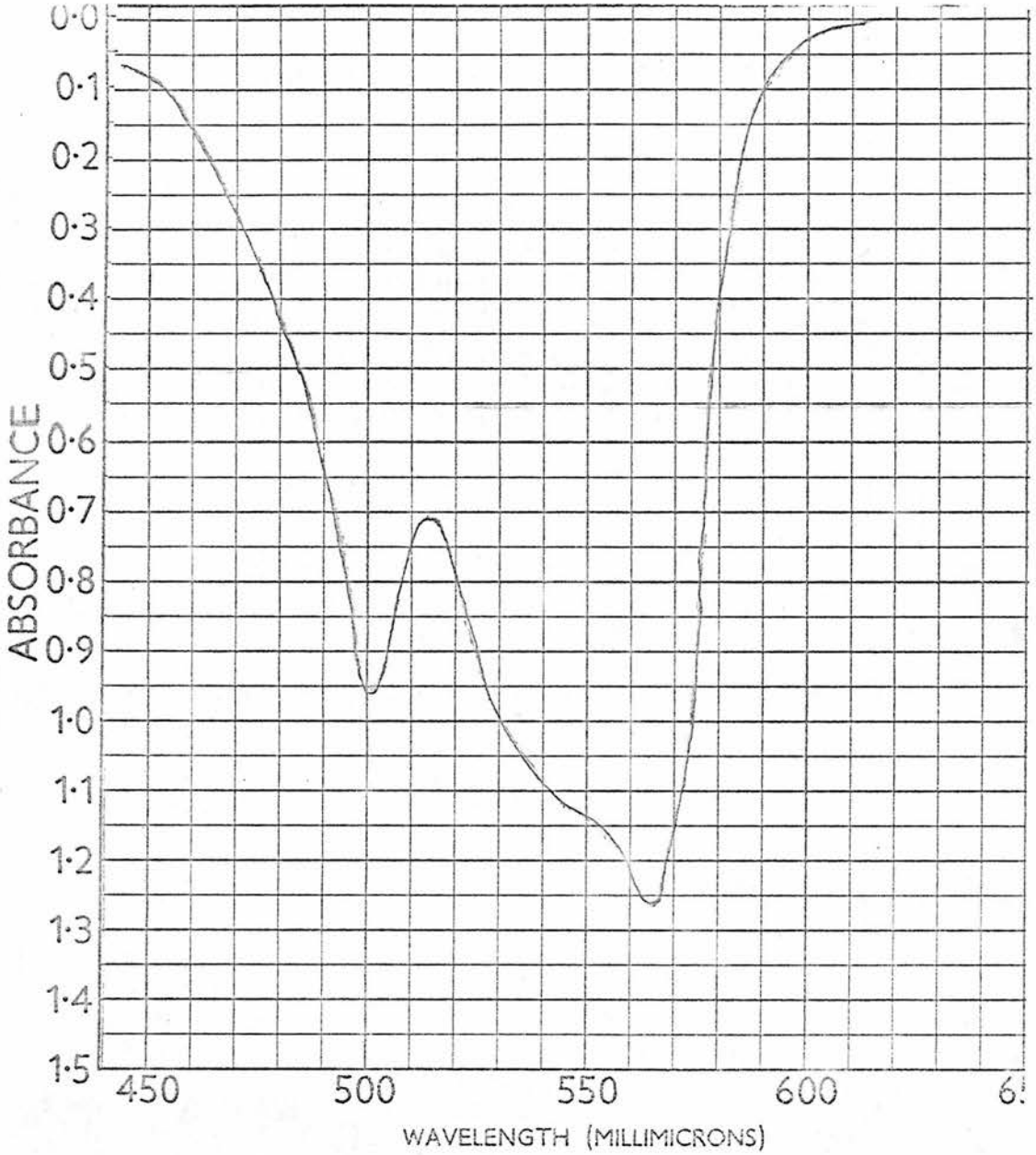


FIGURE X STANDARD PHYCOERYTHRIN AFTER CHROMATOGRAPHY .

and was applied to a column of G-25 Sephadex (35 cm. x 5 cm.) in 5 ml. aliquots. The leading phycoerythrin bands which were free from phycocyanin, rivanol contamination and subunits of phycoerythrin were combined. The spectrum of this standard sample is shown in Figure X. The ratio of the absorption at 495 mμ to that at 275 mμ was used as a secondary criterion of purity ($\frac{1.38}{0.55}$), i.e. 2.5. This standard sample was freeze-dried and was stored as usual in this freeze-dried state.

Since rivanol in solution had been absorbed on kaolin acid clay prior to precipitation of Fraction C, the same method was applied in an attempt to remove contaminating rivanol from Fraction D. A sample of Fraction D (200 mg.) was dissolved in distilled water (500 ml.) and centrifuged (1,800 r.p.m. for 30 mins. at $0^{\circ}C$) and the centrifugate filtered through a celite pad. This filtrate was still purple pink in colour and although its visible spectrum very closely resembled that of standard phycoerythrin its fluorescence was quenched indicating that it was still contaminated with rivanol. Although kaolin has a capacity for absorption of rivanol in free solution its affinity for rivanol was not sufficiently strong enough to cleave this rivanol-phycoerythrin symplex in Fraction D.

It had been observed during the preparation of the standard sample that rivanol was chromatographically separable from phycoerythrin on Sephadex G-75 and on calcium phosphate/ celite. The rivanol was absorbed on the column upper surface and was not eluted by McIlvaine Buffers. A batchwise procedure was attempted following the same general method as

described for kaolin. It was found that the rivanol symplex could not be broken down by Sephadex nor calcium phosphate/celite. However, the calcium phosphate/celite did indicate that Fraction D was not entirely free from traces of phycocyanin. Fraction D (200 mg.) was dissolved in a solution of sodium chloride (1%) and was mixed with a slurry of calcium phosphate/celite (c.a. 10 g.). Washing this slurry with McIlvaine Buffer (0.01 M, pH = 6.5) released phycoerythrin which was still slightly contaminated with rivanol. However, further washings of the slurry with McIlvaine Buffer (0.1 M, pH = 6.5) released a small amount of blue coloured material which, on investigation of its visible spectrum, proved to be phycocyanin. The remainder of Fraction D was submitted to this batchwise treatment with calcium phosphate/celite to give Fractions F (phycoerythrin) and G (phycocyanin).

The most successful means of removal of rivanol without simultaneous denaturation of phycoerythrin was developed from the observation that after dialysis of a rivanol symplex the dialysis bags became quite badly stained with rivanol and a dialysis procedure involving frequent changes of membrane resulted in a fluorescing phycoerythrin. An ammonium sulphate precipitate of the rivanol-phycoerythrin symplex Fraction F was dialysed in an open dialysis bag in the presence of several small strips of dialysis membrane and under continuous stirring. The rivanol was absorbed on the strips of membrane as well as on the bagging itself.

The process could be speeded up by dialysing against a

stirred neutral mixture of ion exchange resins (IR 120 and IR 45) in distilled water (10 L, 0°C) in preference to running water. After two days the non-dialysable fraction was centrifuged and the solution dialysed in sealed bags against running water (0°C for two days). The protein obtained by this method was a free-fluorescing, spectrally pure phycoerythrin which was homogeneous on chromatography (G-75 Sephadex, calcium phosphate/celite).

A second extraction and fractionation of the biliproteins was made from the same source at the Spring low tide. The general extraction and purification procedure as described previously was followed with minor amendments. On this second extraction the algae was cut into much smaller fragments using a supersonic cutter (Ultra Turrax), and the protein content was leached into a solution of sucrose (1%) which enabled complete extraction in two immersions (2 x 2 days) (20 L). The biliproteins were separated on the lines of the most successful fractionation scheme described for the first extraction which is detailed in Tables 8-9 and Figures 17-18. Using this fractionation technique 8.3 g. phycoerythrin and 1.5 g. phycocyanin were extracted from 32 L of freshly harvested alga.

It was discovered that freeze-dried samples of phycoerythrin contained a certain percentage of insoluble material and it was suggested that this was denatured phycoerythrin. By freeze-drying a sample of phycoerythrin, weighing this sample, redissolving in water, centrifuging and freeze-drying the soluble and insoluble fractions it was estimated that this

insoluble portion constituted 15% of the original phycoerythrin.

Small scale hydrolysis with hydrochloric acid (G.P. 9, B) and subsequent chromatographic identification of the amino acids (G.P. 2) indicated that the insoluble and soluble fractions contained the same amino acids as R-phycoerythrin. Small scale resin hydrolysis (100°C) of the soluble and insoluble portions (G.P. 15) and chromatographic examination of the sugars in the water wash (G.P. 1, A) indicated that the insoluble portion contained the same sugars in approximately the same amounts as the soluble portion.

Hydrolysis of the soluble and insoluble fractions with concentrated hydrochloric acid and examination of the visible spectra of the chloroform soluble phycobilins obtained indicated that both soluble and insoluble fractions released phycoerythrobilin which exhibited an absorption maximum at 570 mμ.

On the basis of this evidence we can assume that this insoluble portion which arises on freeze-drying is denatured R-phycoerythrin.

BILIPROTEIN EXTRACTION AND FRACTIONATION SCHEME . (A)

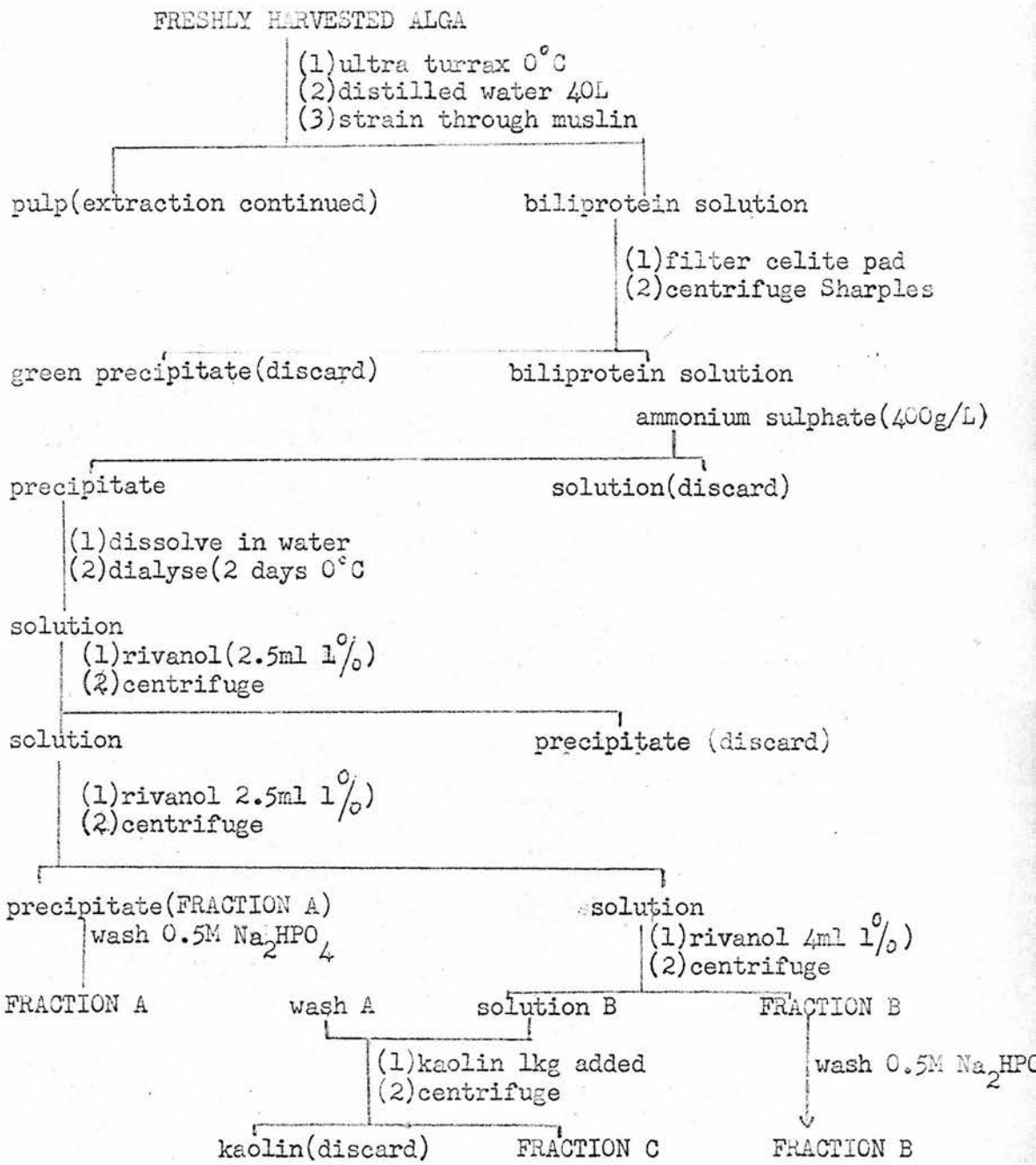


TABLE 8 .

BILIPROTEIN EXTRACTION AND FRACTIONATION SCHEME . (B)

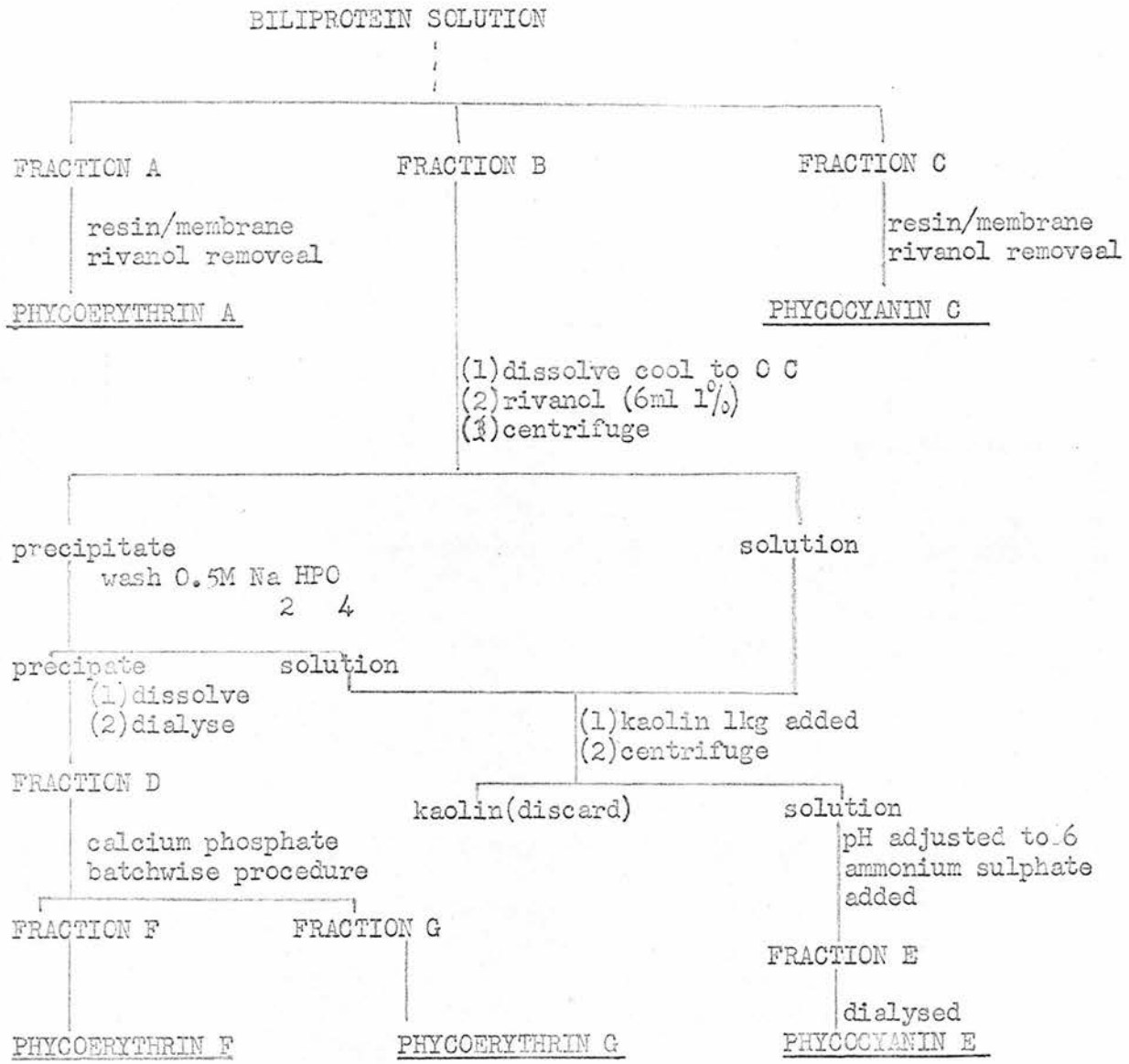


TABLE 9.

EXPERIMENTAL SECTION 1

Direct carbohydrate investigation

The carbohydrate found in phycoerythrin by Fujiwara[45] and Tsuchiya[46] was identified by hydrolysis of the native protein, fractionation of the constituents using ion exchange resins, and investigation of the neutral portion by paper chromatography. The low percentage of carbohydrate present in this chromo-glyco-protein precludes other more rigorous means of identification and in this work the techniques of paper and thin layer chromatography as described in the General Methods section were adopted as those most suitable.

Initially a series of small-scale resin hydrolyses (G.P. 15, 100°C) were carried out on portions of phycoerythrin (50 mg.). A comparison of results revealed that the same components in roughly the same relative proportions were present in the November phycoerythrin and in the May phycoerythrin.

Examination of the carbohydrate components of samples of phycoerythrin (50 mg.) which had been subjected to calcium phosphate chromatography (G.P. 16), Sephadex chromatography (G.P. 17) and prolonged dialysis and of a sample subjected to all these procedures revealed that the samples contained the same components in roughly the same proportions.

The hydrolysate from the resin procedure was divided into two fractions: a neutral wash and an acid wash. Investigation of the acid wash indicated that it contained no carbo-

hydrate. The carbohydrate of the neutral wash was examined by paper and thin layer chromatography (G.P. 1, A and B; G.P. 5, 2). The sugars present in the native protein were identified as glucose (+++), galactose (++), arabinose (++), xylose (+) and rhamnose (trace). Some slower moving, oligosaccharide material could be located by the more sensitive development reagents. The R_f and the R_{gal} values for these components suggested that they were glucose- or galactose-containing disaccharides. A further trace spot could be located which was thought to be fucose. Further investigation failed to establish that it definitely was fucose.

The neutral wash was investigated for acetylated amino sugars. No acetylated amino sugars could be located using the specific Partridge reagent (G.P. 1, IV) or ninhydrin spray reagent (G.P. 1, III). Comparison of the R_f and R_{gal} values of the more common amino sugars indicated that none of the components located by silver nitrate dip (G.P. 1, II) corresponded to acetylated amino sugars.

The acid wash from the resin hydrolysate (G.P. 9, B) was deionised and the hydrolysate examined by paper chromatography (G.P. 1, A, B, C and D). No amino sugars could be located. A further sample of this deionised acid fraction was chromatographed on Dowex 50 Wx8 (H^+) (eluted with 4 $\underline{N}$HCl). Investigation of the eluate indicated that no amino sugars were present.

Careful investigation of the resin hydrolysates indicated that no sulphate sugars were present as bound carbohydrate.

A sample of phycoerythrin (50 mg.) was hydrolysed with

sulphuric acid (G.P. 9, A). The hydrolysate was neutralised with barium hydroxide and barium carbonate and was deionised. The neutral fraction was investigated by paper chromatography and the carbohydrate components were identified as glucose (+++), galactose (++), xylose (+), arabinose (++) and rhamnose (trace). This agreed with the resin hydrolysis results.

Several attempts were made to estimate the percentage of bound carbohydrate present in phycoerythrin. A series of carbohydrate estimations by means of the phenol/sulphuric acid method and the p-aminobenzoic acid method were performed on freeze-dried samples of phycoerythrin. No consistent results could be obtained and it was deduced that the chromophore absorptions were interfering with the colorimetry. An attempt to combat this interference of the chromophore was made. The protein samples were digested overnight at 38°C with trypsin (1% w/w) and pronase (1% w/w). Samples of the digest were submitted to phenol/sulphuric acid colorimetry against enzyme blanks. It was estimated that the protein contained 4.5 - 5.5% carbohydrate.

A weighed portion of phycoerythrin (100 mg.) was resin-hydrolysed for 72 hours at 100°C (G.P. 15), conditions which were found to give complete hydrolysis. The water wash was reduced in volume to dryness and was taken up in water (50 ml.). Aliquots of this water wash were subjected to the carbohydrate estimation techniques. The phenol/sulphuric acid method indicated that phycoerythrin contained 4.6 - 4.8% carbohydrate. The p-aminobenzoic acid method indicated that the protein

contained 3.1 - 3.6% hexose and 1.3 - 1.5% pentose.

A further method was employed to obtain a more accurate hexose to pentose ratio. Half the resin hydrolysate water wash obtained as described previously was submitted to quantitative paper chromatography. The sample was chromatographed on Whatman No. 1 paper for 48 hours in Solvent D. The separated hexoses and pentoses were eluted from the chromatograms. The percentage sugar was estimated by the standard methods against paper wash blanks. The phenol/sulphuric acid technique gave a hexose/pentose figure of 2.0 ± 0.1 and the p-aminobenzoic acid technique a figure of 2.0 ± 0.1.

The percentage carbohydrate in native phycoerythrin was also estimated by colorimetric determinations performed on deionised acid hydrolysates. Samples of phycoerythrin were hydrolysed (G.P. 9, B), the hydrolysates were reduced in volume to 2 ml. and passed through a column of carefully regenerated Dowex 50 W x 8. The colourless eluate (15 ml.) was reduced in volume to 2 ml. and the carbohydrate content estimated by phenol/sulphuric acid colorimetry against resin eluate blanks. High resin blanks could only be avoided by using freshly regenerated resin in darkened columns. The carbohydrate content of native phycoerythrin was estimated by this method as 6.5 - 7.5%.

Low temperature resin hydrolysis

Previous resin hydrolyses performed at 100°C gave some very tentative evidence for the presence of oligosaccharide material in phycoerythrin. Although the resin hydrolysis procedure is not a rigorous hydrolysis procedure it is effective enough at 100°C to hydrolyse carbohydrate-carbohydrate linkages as well as amino-acid-carbohydrate linkages. It was expected therefore that using a milder resin hydrolysis technique (i.e. at 60°C) it might be possible to promote the latter hydrolysis without a second stage oligosaccharide hydrolysis occurring and hence increase the yield of oligosaccharide.

A series of low temperature resin hydrolyses were carried out. The water washes were investigated by paper chromatography and were found to contain glucose (+++), galactose (++), arabinose (++) and rhamnose (trace) in relatively the same proportions as before. There seemed to be a slight increase in the amount of oligosaccharide material produced. Hydrolysis of the water wash with sulphuric acid resulted in disappearance of the oligosaccharide spot as did digestion with almond emulsin. A series of attempts to isolate and characterise this oligosaccharide involving quantitative paper separation (Whatman No. 1, G.P. 1, B), methylation and methanolysis failed. Attempts were made to separate the oligosaccharide material on Dowex 50 Wx8 (Li^+) columns were also unsuccessful. The complexity of the monosaccharide portion and the small amounts of oligosaccharide involved made the problem very difficult.

Peptides hydrolysed by Trypsin

	Rate of Hydrolysis
Bz - Arg - NH_2	++
Ts - Arg - NH_2	+++
Bz - Arg - OMe	++++++
Ts - Arg - OMe	+++++
Bz - Arg - OEt	++++++
Bz - Lys - NH_2	++
Lys - OEt	++++
Bz - Gly - Arg - NH_2	++++
Gly - Lys - NH_2	+
Bz - Gly - Lys - NH_2	++++
Lys - Lys - NH_2	+
Lys - Lys - Lys	+
Bz - Lys - Lys - Lys	++++

FIGURE 19. TRYPTIC SPECIFICITY.

EXPERIMENTAL SECTION 2

Tryptic Hydrolysis

Selective degradation of proteins by proteolytic enzymes, which converts protein into peptides, has been used to give an insight into protein structure. Proteolytic hydrolysis of a glycoprotein and subsequent separation of the resulting peptides gives an indication of the number of points of carbohydrate attachment to the polypeptide core and at the same time this separation may result in the isolation of a glycopeptide more structurally informative than the original glycoprotein, i.e., one which has a higher percentage of carbohydrate and fewer amino-acid constituents. Ideally, proteolysis with a series of enzymes could result in a glycopeptide consisting of a single amino-acid linked to an oligosaccharide unit since the bond between amino-acid and carbohydrate should be resistant to proteolytic attack. Several authors have employed proteolytic enzymes in the structural investigation of glycoproteins.

Trypsin is excreted by the pancreas and is an exceedingly active proteolytic enzyme which is very stable; it can be heated to boiling without losing its activity. The tryptic reaction on synthetic substrates follows the kinetics of a first order reaction. The endopeptidase trypsin, in common with most proteolytic enzymes, attacks linkages activated by certain neighbouring or participating amino-acids (Figure 19). Trypsin has been employed in the proteolytic hydrolysis of fetuin, γ-globulins, ovomucoid and ovalbumin. Fujiwara

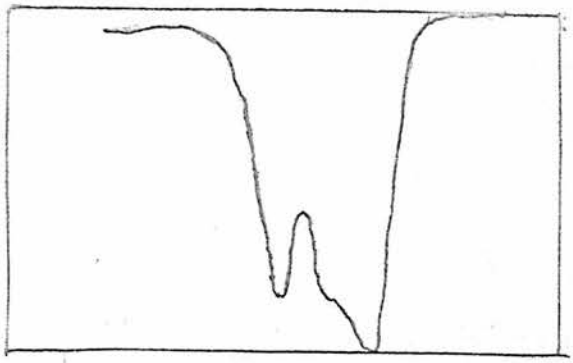
described digestion of phycoerythrin with trypsin in an attempt to isolate a core chromopeptide and the tryptic digestion technique described here was developed from her results.

Small-scale Hydrolysis and Preliminary Fractionation

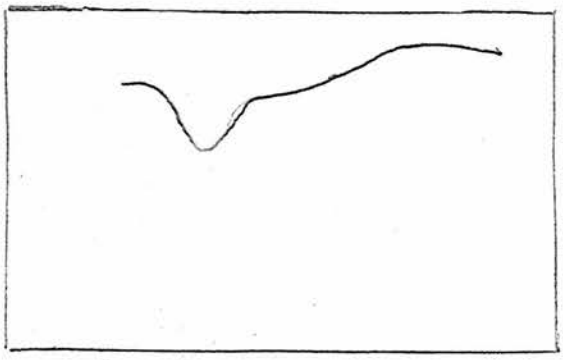
Small-scale tryptic hydrolyses were carried out in narrow necked, conical flasks (250 ml.). The flasks were cleaned with chromic acid and detergent and were sterilized in a pressure cooker (15 lb./in.^{2}, 30 min.). A known weight of protein was dissolved in water or McIlvaine Buffer (phosphate/citrate, pH 7, 0.01 M). The volume was made up to 100 ml. The enzyme (1% w/w) was added and the pH adjusted to 7. The liquid surface was covered with a layer of toluene (5 ml.) and a wad of cotton wool was placed in the neck of the flask. The flasks were placed in an oven at 37°C and were incubated for 48 hours. The release of acid by the digest was measured at regular intervals (20 min.) and the pH adjusted to 7 by the addition of dilute ammonia (0.1 M). After 24 hours a further addition of enzyme (1% w/w) was made.

The pH of the digest was finally adjusted to 7 and after separating the toluene layer the digests were centrifuged (2,000 r.p.m. for 20 min. at 0°C). The solution was then reduced in volume (10 ml.) and centrifuged again (3,000 r.p.m. for 20 min. at 0°C).

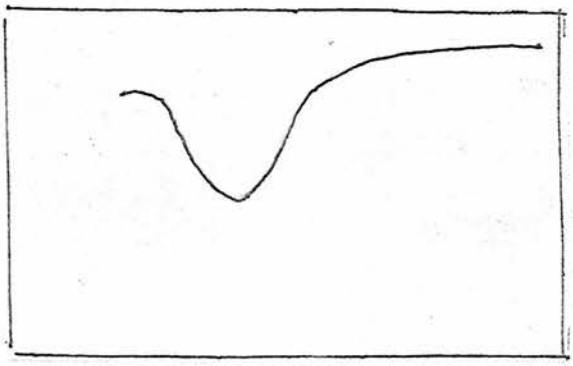
Phycoerythrin (Extr. 1, 200 mg.) was digested with trypsin and the digest finally reduced in volume and centrifuged. The green precipitate (A) was freeze-dried. The digest solution was then chromatographed on G-25 Sephadex (G.P. 17) in water and 2 ml. fractions were collected. The visible spectra of the fractions were taken and tubes of like scan were bulked. The chromatography resulted in four main bands - the leading a



BAND B

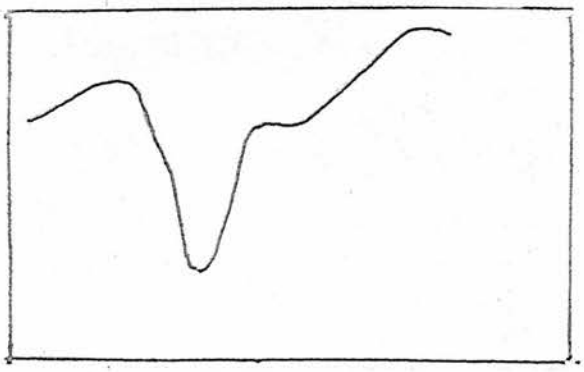


BAND C

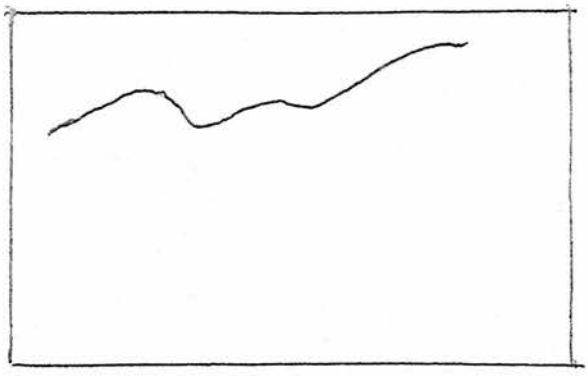


BAND D

FIGURE 20 SMALL SCALE TRYPTIC DIGESTION - SPECTRAL BANDS



BAND A



BAND B

FIGURE 22LARGE SCALE TRYPTIC DIGESTION - SPECTRAL BANDS

pink band (B), the second a red band (C), the third brown (D) and the final band colourless (E). The visible absorption spectra of the bands is given in Figure 20. The bands were freeze-dried and were stored in stoppered vials.

The experiment was repeated using a different trypsin preparation (Light and Co.) and it was found that this gave a lesser proportion of Band A to Bands B, C, D and E and that the release of acid was much greater in the early stages of digestion. There was also a marked decrease in the amount of Band A obtained. In all the succeeding experiments this trypsin preparation (Light and Co.) was used.

It was suspected that Band B was either unchanged phycoerythrin or some subunit of phycoerythrin. The patterns obtained by ultracentrifugation of Band B and a soluble sample of phycoerythrin indicated that Band B was of the same order of molecular weight as phycoerythrin and not a subunit. A sample of Band B was redigested with trypsin (24 hours). The digest was reduced in volume and chromatographed as before (G-25 Sephadex). This chromatography resulted in a fractionation of the digest into three bands - a red band, a brown, and a colourless band. Investigation of the visible spectra of these bands indicated that they were identical to Bands C, D and E obtained previously. It can be concluded that Band B is phycoerythrin which was not digested on the original tryptic hydrolysis (Table 10).

Band A was investigated after acid hydrolysis (6 N Hcl) and resin hydrolysis (G.P. 15), and from a comparison of the

Table 10A. Tryptic Digestion - Small Scale Scheme

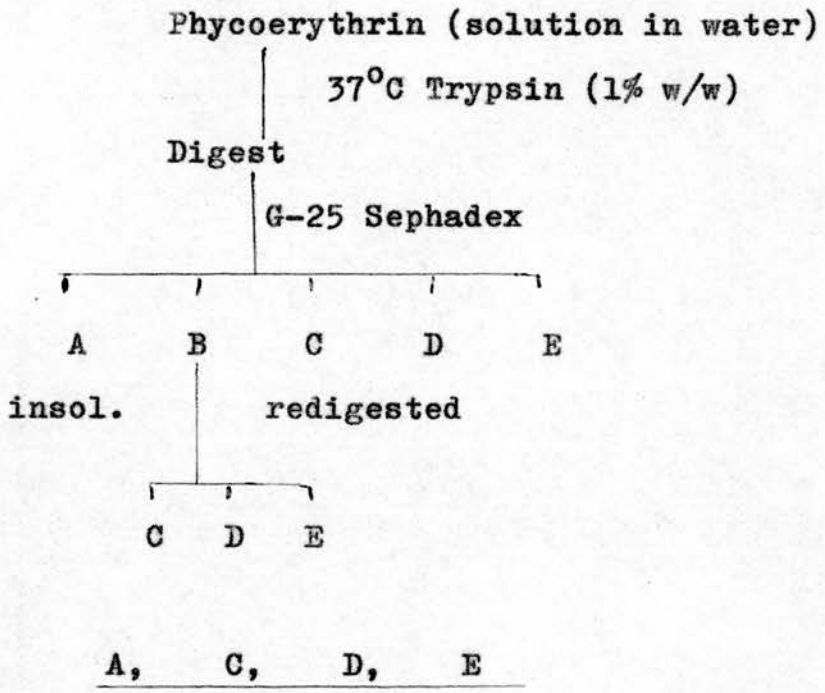
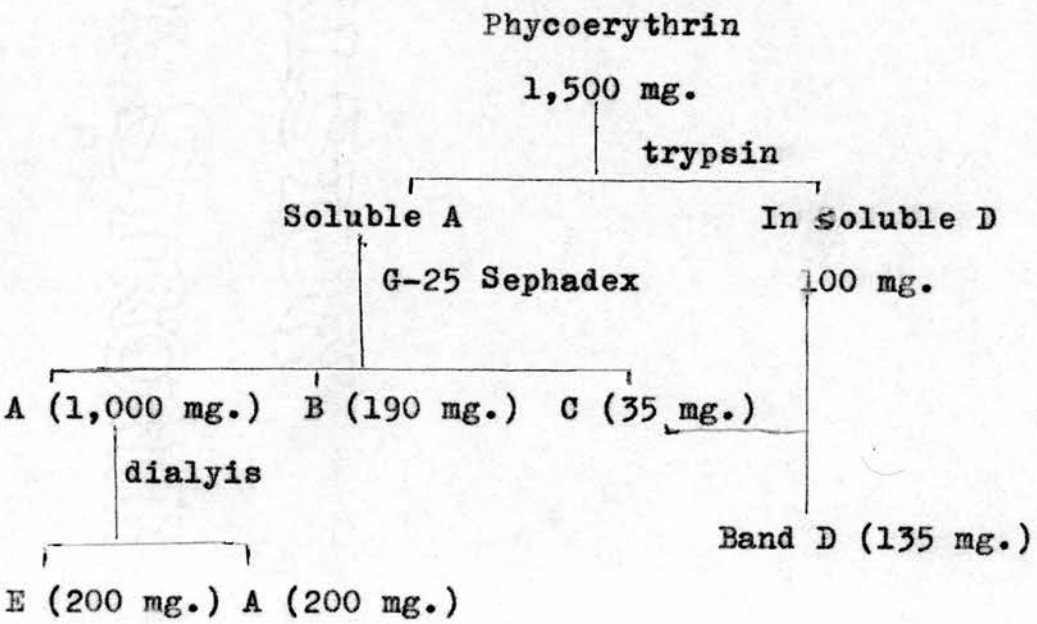


Table 10B. Tryptic Digestion - Large Scale Scheme



results from these hydrolyses and similar hydrolyses of denatured phycoerythrin it was concluded that Band A was denatured phycoerythrin. Tryptic digestion of a centrifuged, soluble preparation of phycoerythrin resulted in a decrease in the amount of Band A produced, providing further evidence for this conclusion.

Phycoerythrin (200 mg., Extr. 2) was digested with trypsin under the same conditions as those employed for the Extraction 1. Phycoerythrin and the same fractions in the same relative amounts were obtained. The combined Bands B were redigested and the resultant Bands C, D and E were combined with the relative Bands C, D and E from the previous experiments. Bands C, D and E were freeze-dried and samples (5 mg.) of the bands were subjected to acid hydrolysis and investigation of the same amino acids present. Two-dimensional chromatography indicated that Bands C and D contained all the amino-acids present in R-phycoerythrin (G.P. 2). Investigation of the basic amino acids by exchange paper chromatography (pH 5.2, McIlvaine Buffer, Amberlite LA2 paper) indicated that histidine was present in both these fractions (histidine is the minor amino-acid component of phycoerythrin). Band E contained only trace amounts of amino acid constituents. Samples of Bands C, D and E (5 mg.) were resin hydrolysed (100°C) (G.P. 15) and the water washes investigated by paper chromatography (G.P. 1). C was found to contain carbohydrate which was chromatographically identical in two solvents (A and C) to galactose, glucose, arabinose and rhamnose. E contained xylose and arabinose.

Band E, which proved to be largely salt, was investigated directly for carbohydrate by running a sample which had not been hydrolysed in the normal chromatographic solvents. Results from this also indicated that the arabinose and xylose in Band E were not bound to any amino-acid but had been freed as a result of tryptic digestion. An attempt to repeat this result by chromatography of a small-scale digest of phycoerythrin failed. Examination of all the colourless eluate showed that no free sugars were present. The carbohydrate content of the bands was measured by subjecting weighed, freeze-dried samples of the bands to sugar estimation by the phenol/sulphuric acid colorimetric method (G.P. 13). Results indicated that whereas native phycoerythrin could be calculated to contain 7.5% carbohydrate by this method, Band C contained 8.5%, Band D 4.5% and Band E 2.5%. Bands C and D were considered most interesting for further studies and Band E was regarded as an impure, salt-contaminated fraction. Bands C and D were submitted to electrolysis in a Wieland-Fischer type apparatus (G.P. 3A). Preliminary experiments showed that C and D could be resolved electrophoretically into five or six main electropositive components which fell into two groups and which could be located by ninhydrin (G.P. 1, III). Trial electrophoresis in phthalate buffer, pyridine : acetic acid : water and a solution of acetic acid (5%) indicated that the last named gave the clearest separation. Electrophoresis in that buffer (450 v, 22.5 v/cm. for 45 min.) resolved C into two main bands, one containing six components and the other two.

Band D could be resolved into two main bands, one containing five components and the other two.

Band C (10% ACOH, 22.5 v/cm., 45 min.)

α_C (1.9 cm., 2.25 cm., 2.85 cm., 3.45 cm., 4.05 cm., 4.60 cm.)

β_C (5.45 cm., 6.10 cm.)

Band D (10% ACOH, 22.5 v/cm., 45 min.)

α_D (1.80 cm., 2.20 cm., 2.70 cm., 3.40 cm., 3.90 cm.)

β_D (5.50 cm., 5.90 cm.)

Continuous Electrophoresis

Since Bands C and D could be further resolved into several components by paper electrophoresis in 5% aqueous acetic acid it was decided to submit the bands to preparative electrophoresis before further examination of the resolved bands. The preparative apparatus used was of the curtain electrophoresis type - the Beckman-Spinco C.P. continuous flow electrophoresis cell. The voltage was developed by the C.P.D. constat regulated power supply and the fractions were collected using the model C.P.F. fraction collector. An electric field across a hanging paper curtain deflects the electrically charged components of the sample towards the side of opposite charge, causing different mobility classes to follow different paths down the curtain. The sample was fed continuously to the paper curtain and joined a uniform flow of electrolyte down the curtain. The sample streams were collected in test tubes placed directly beneath 32 tabs notched into the bottom

of the paper curtain. An automatic fraction collector was used during long runs. Separation could be controlled by varying the feed rate of the sample and the flow rate of the background electrolyte as well as the field strength. After fixing these three variables the continuous sample feed enabled long uniform runs under reproducible conditions. The electrophoresis cell was equilibrated with the acetic acid solution (5%) for twenty-four hours before each run. The flow over the curtain was checked before each run using dye spots and was adjusted till vertical with no deviations in flow towards the wicks. The sample (Band C dissolved in 50 ml. 5% acetic acid) was applied at Feed Tab No. 4, i.e., nearest the positive electrode. The sample feed was set at 2 ml. per hour. The constat was adjusted to give a constant voltage (500 v.). Fractions were collected over 32 tubes and the automatic fraction collector set at 6 hour intervals. Solvent flow was set at 12-14 cm./hr.

Prior to the continuous electrophoresis, trials in the Wieland-Fischer apparatus were carried out using the Schleider and Schuell 470 electrophoresis paper which is recommended for the hanging curtain. These trials indicated that the conditions described previously would be effective. The experiment was repeated with sample Band D (dissolved in 50 ml. 5% acetic acid).

Investigation of Electrophoresis Fractions

1) Band C. The contents of each tube were reduced in volume to near dryness and two samples were taken from each tube. A

Table 11. Investigation of carbohydrate in CE_{1-5}

Sample	% carbo-hydrate	Carbohydrate constituents
CE_1	8.5%	galactose (++) glucose (+++) arabinose (+)
CE_3	7.5%	glucose (+++) arabinose (+) rhamnose (tr.)
CE_4	6.0%	glucose (+++) arabinose (+)
CE_5	8.5%	glucose (+++) arabinose (++) galactose (tr.) slower moving spot; R_g = 0.62 in Solvent A

Table 12. Amino acids in Bands CE_{1-5}

Amino acid	CE_1	CE_3	CE_4	CE_5
aspartic	+++	++	++	+
threonine	+	+	+	-
serine	tr.	tr.	tr.	-
glutamic	+++	++	+	+
proline	++	-	-	-
glycine	+	+	-	+
alanine	++	+++	++	++
valine	++	+	+	+
methionine	+	+	+	+
isoleucine	+	+	+	-
leucine	+	+	+	-
tyrosine	+	+	+	-
phenylalanine	+	+	-	-
lysine	+++	+	++	+
histidine	++	+	++	+
arginine	+	-	++	+

sample was subjected to electrophoresis on paper in the Wieland-Fischer apparatus (5% AcOH, 450 v., 45 min.) and a second sample to paper chromatography (G.P. 1, D). The chromatograms and electrophoretograms were developed with ninhydrin (G.P. 1, III). As a result of this examination the tubes were bulked (electrophoretically and chromatographically like tubes were combined) and Band C was resolved into five bands, CE_1, CE_2, CE_3, CE_4, CE_5.

2) Band D. Investigation of the electrophoretic fractions of Band D followed the same pattern as investigation of Band C fractions. As a result of this examination the tubes were bulked as before. Band D was resolved into three main fractions, DE_1, DE_2, DE_3. DE_2 and DE_3 proved chromatographically and electrophoretically identical to CE_3 and CE_4 and were combined with them.

Band C	CE_1	CE_2	CE_3	CE_4	CE_5
Band D	DE_1		DE_3	DE_4	

Glycopeptide Examination

The carbohydrate and amino acid compositions of Bands CE_{1-5} were investigated using the resin hydrolysis technique. The samples (5 mg.) were resin hydrolysed for 24 hours at 100°C. The water washes were examined for carbohydrate by phenol/sulphuric acid colorimetric determination (G.P. 14, A) and by chromatography (G.P. 1, A). Chromatograms were developed with p-anisidine spray reagent and silver nitrate/sodium hydroxide dip reagent (G.P. 1, I and II). The results are shown in Table 11.

The acid washes from the resin hydrolysates were reduced in volume to dryness and were further hydrolysed (6 N HCl for 24 hrs.). The hydrolysates were desalted and the amino-acid composition determined chromatographically (G.P. 2, A) (Table 12).

Glycopeptide Band CE_5

Band CE_5 was the most interesting of the fractions investigated since it contained only nine of the original amino-acids present and since resin hydrolysis revealed the presence of what appeared to be an oligosaccharide. A further portion (5 mg.) of CE_5 was submitted to resin hydrolysis and the carbohydrate investigated by paper chromatography (G.P. 1, A) and thin layer chromatography (G.P. 5). The results confirmed that the band contained glucose, arabinose, galactose and a slower moving spot. Thin layer chromatography indicated that this spot was cellobiose (G.P. 5, 3). The possibility that the carbohydrate in Band CE_5 was an artefact from the electrophoresis paper was investigated. Samples of the band were spotted directly, without hydrolysis, and were chromatographed on paper (G.P. 1, A and D) and thin layer (G.P. 5, 3). No free carbohydrate was located. Band CE_5 was a syrup and could not be freeze-dried to give a powder. To remove impurities and verify the fact that the carbohydrate was peptide bound the band was chromatographed on Dowex 50wx2 (H^+). Freshly regenerated resin was packed in a column (15 cm. by $\frac{1}{2}$ cm.) and was washed exhaustively with water. The sample was introduced

to the column in aqueous solution and the wash collected in 10 ml. fractions. No carbohydrate could be found in the water wash. The column was then washed with dilute ammonia solution (2 N). The eluate was neutralised with dilute acetic acid (1 N) and was freeze-dried. A sample of this fraction was resin hydrolysed (G.P. 15) at 100°C and the carbohydrate and amino acid components examined chromatographically. Results were identical to those obtained from hydrolysis of Band CE_5 prior to Dowex chromatography. The substrate specific enzymes, maltase and almond emulsin, were used to determine whether the disaccharide was cellobiose or maltase. Samples of the Dowex chromatographed Band CE_5 were resin hydrolysed and the water washes were incubated (37°C) with samples of almond emulsin and maltase. The digests were reduced in volume and were investigated by thin layer chromatography (G.P. 5, 3). Digestion of the band with almond emulsin resulted in disappearance of the disaccharide accompanied by release of glucose. This gives further indication that the disaccharide was cellobiose. Digestion of the sample with maltase resulted in no change. Further verification of the disaccharide structure by methylation of the intact glycopeptide Band CE_5 followed by methanolysis (G.P. 13) and gas-liquid chromatography (G.P. 14) was attempted but the results were inconclusive. 2,3,4,6-tetramethyl glucose could be located, probably arising from the glucose sample, but no trimethyl methyl glucosides could be located. Although two-dimensional paper chromatography-electrophoresis indicated that Band CE_5 contained four major

components, which could probably be resolved chromatographically, the results give some insight into the nature of this group of glycopeptides.

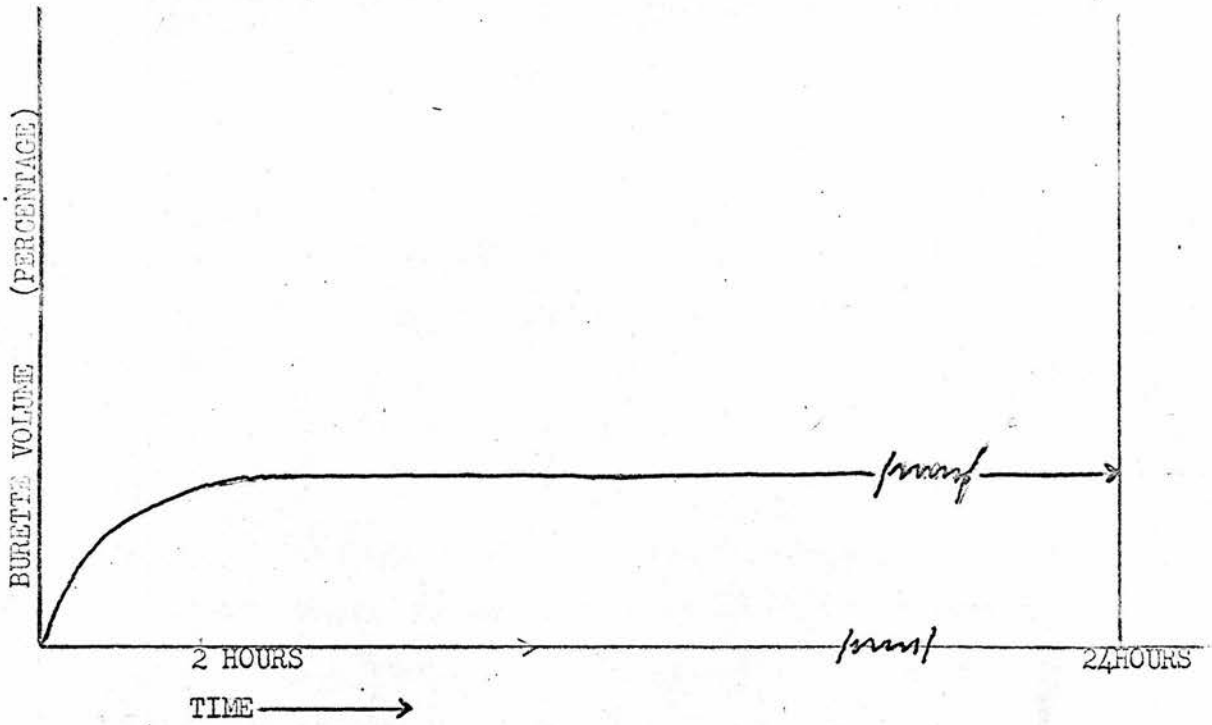


FIGURE 21 UPTAKE OF BASE DURING TRYPTIC HYDROLYSIS .

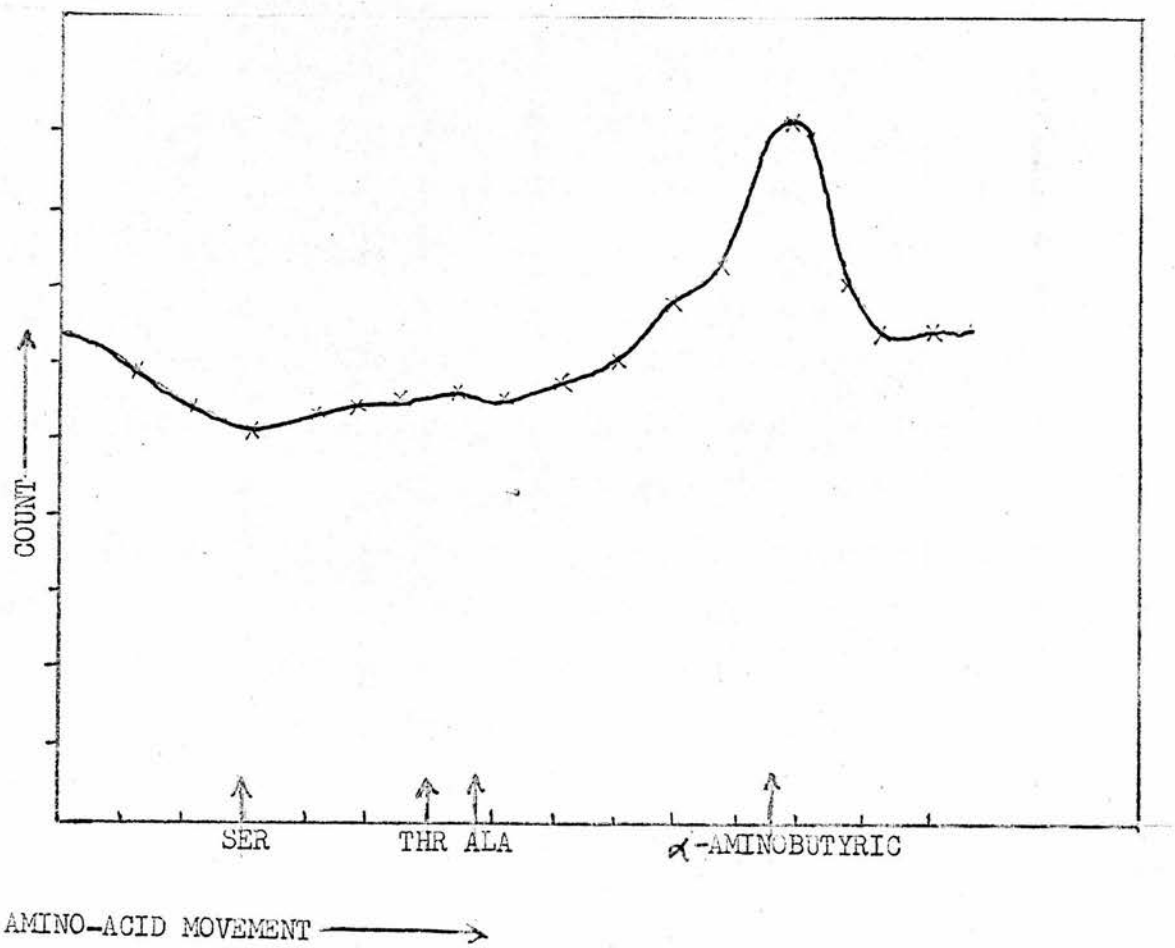


FIGURE 25 AMINO-ACID COUNT AFTER B.W.A. CHROMATOGRAPHY .

Large-scale Tryptic Digestion and Peptide Resolution

In an attempt to isolate a glycopeptide which was homogeneous to electrophoresis and chromatography and which would be more useful for structural studies a large-scale digestion of phycoerythrin with trypsin was carried out. The procedure which is detailed in the following pages is not a rigorous one involving complete fractionation of all the components but is a procedure which was dictated by the results of two-dimensional paper and thin layer electrophoresis-chromatography, the quantities of the fractions involved, the amino-acid and carbohydrate composition and the actual percentage of carbohydrate in the fraction with the primary aim being the isolation of a glycopeptide which was homogeneous and contained a high percentage of carbohydrate.

Digestion and Preliminary Fractionation

Portions of phycoerythrin (3 x 500 mg.) were digested with trypsin (1% w/w) in aqueous solution at pH 7 and at 38°C under nitrogen. The digests were slowly stirred throughout the reaction. The pH was maintained by the addition of dilute sodium hydroxide (0.1 N) by means of a pH-stat controller (Radiometer, Copenhagen). The amount of acid released (equivalent to the amount of base required for neutralisation) was recorded for each digestion on the automatic recorder. After a period of 24 hours, by which time the release of acid had ceased, a further addition of enzyme was made. Figure 21 shows the uptake of base recorded against a time base. The

amount of acid released on digestion of one portion of the protein (500 mg.) was calculated as equivalent to 1 ml. of 0.1 N sodium hydroxide, i.e., 0.100 milli equivalents. From this it can be calculated that the average molecular weight of the peptides obtained is in the region 500 - 1,000.

After digestion the digests were reduced in volume to 40 ml. (G.P. 8), centrifuged (1,800 r.p.m., 30 min., 0°C) and the precipitate washed with water (10 ml.). The combined centrifugate and washings were then chromatographed on G-25 Sephadex and the insoluble portion (D) was freeze-dried.

The soluble fraction was made 0.1 M with respect to acetic acid and was introduced to a column of G-25 Sephadex (3 cm. x 35 cm.) (G.P. 17) which had been equilibrated with 0.1 M acetic acid and which was eluted with the same solvent. The eluate was collected in 5 ml. fractions. Samples of each fraction were subjected to hydrolysis (G.P. 9, B) and direct ninhydrin estimation (G.P. 18) and a plot of amino-acid content against tube number was made. The visible absorption spectrum of the solution in each tube was taken against acetic acid blanks. From the amino-acid content curve and the visible spectrum of the fractions it was decided to bulk the eluate in two main fractions, Fraction A, the faster moving fraction component which exhibited a strong absorption maximum at 495 mμ and a shoulder at 550 mμ, and Fraction B which exhibited absorption maxima of almost equal intensity at 495 mμ and 560 mμ (Figure 22).

A green band, Band C, was eluted from the column with

Table 13. Amino-acid constituents in Bands A - E

Amino acids	A	B	C	D	E	Phycoerythrin
aspartic	+++	+++	++	++	+++	10 +
threonine	++	-	-	-	++	4 +
serine	++	++	+	+	+	7 +
glutamic	+++	++	+	+	+	7 +
proline	+	+	-	-	+	3 +
glycine	++	++	++	++	++	4 +
alanine	++	++	++	++	+	9 +
valine	+	tr.	+	+	-	6 +
methionine	+	tr.	+	+	+	2 +
isoleucine	+	tr.	+	+	+	4 +
leucine	++	tr.	+	+	+	7 +
tyrosine	-	-	+	+	-	4 +
phenylalanine	+	tr.	+	+	+	3 +
lysine	+	tr.	+	+	+	4 +
histidine	+	tr.	+	+	+	1 +
arginine	++	+	+	+	++	7 +
cystine	-	-	+	+	-	4 +

Table 14. Carbohydrate components in Bands A - E

Phycoerythrin	Band A	Band B	Band C	Band D	Band E
Spot R_g = 0.6	+	+	+	+	+
glucose	+++	+++	++	++	+++
galactose	+	tr.	tr.	tr.	+
arabinose	++	++	+	+	+
xylose	+	+	+	+	+
rhamnose	+	-	-	-	+

molar acetic acid solution. The bulked fractions were neutralised with freshly distilled triethylamine, reduced in volume (G.P. 8) and were finally freeze-dried. Band A would not freeze-dry and was dialysed against distilled water (1 l.) to give Band A (the non-dialysable fraction) and Band E, the dialysable. A summary of this fractionation of the tryptic digest is given in Table 10.

Investigation of the Tryptic Digest

The Bands A, B, C, D and E were investigated by the normal methods. Samples of the bands were hydrolysed (6 N HCl, G.P. 9, B) and the hydrolysates examined by two-dimensional chromatography (G.P. 2). Table 13 shows the amino-acid components of each band compared with the amino-acid components of phycoerythrin. Histidine and the other basic amino-acids were investigated by ion exchange resin paper chromatography (Amberlite LA_2 - Buffer, pH 5.2).

Samples of phycoerythrin, Bands A, B, C and D, were subjected to small-scale resin hydrolysis (G.P. 15) and the neutral wash was chromatographed (G.P. 1, A) and examined for carbohydrate content. The results are shown in Table 14.

Bands A, B, C, D and E were submitted to electrophoresis in the Wieland-Fischer type apparatus (G.P. 3). Preliminary experiments indicated that Bands A and B could be resolved into six components and Band E into two components. Bands D [redacted] could not be resolved under normal conditions.

Band A - 2.0 cm., 2.25 cm., 2.85 cm., 3.5 cm., 5.45 cm., 6.2 cm.

Band B - 2.0 cm., 2.5 cm., 2.9 cm., 3.5 cm., 5.5 cm., 6.2 cm.

Electrophoresis for 45 min. at 22.5 v/cm. in 5% acetic acid.

Continuous Electrophoresis

As a second step in the procedure to isolate a homogeneous glycopeptide it was decided to subject Bands A and B to continuous electrophoresis. Small-scale electrophoreses under various conditions were carried out and the conditions for continuous electrophoresis finally chosen were those adopted for the previous fractionation. It was decided that using 5% acetic acid buffer with a constant potential of 500 V (30-35 ma.) and a solvent flow of 13 cm. per hour, a reasonable separation would be achieved. The sample was applied at the rate of 1 ml. per hour and the sample collector set at a 12 hour collection interval. The cell was equilibrated with buffer for 24 hours previous to each run and each sample required a total running time of 48 hours. Samples were applied in a solution of 5% acetic acid. Both bands were fractionated under these conditions. Band B was fractionated in two parts due to a change of hanging curtain midway through the operation.

Tubes 1-32 from the separate racks were bulked according to number for each experiment, were neutralised with a few drops of freshly distilled triethylamine and were freeze-dried. The three sets of 32 fractions (A, B_1 and B_2) were investigated by two-dimensional electrophoresis-chromatography (G.P. 5, 4)

Table 15A. Electrophoretic Fractionation of Bands A and B

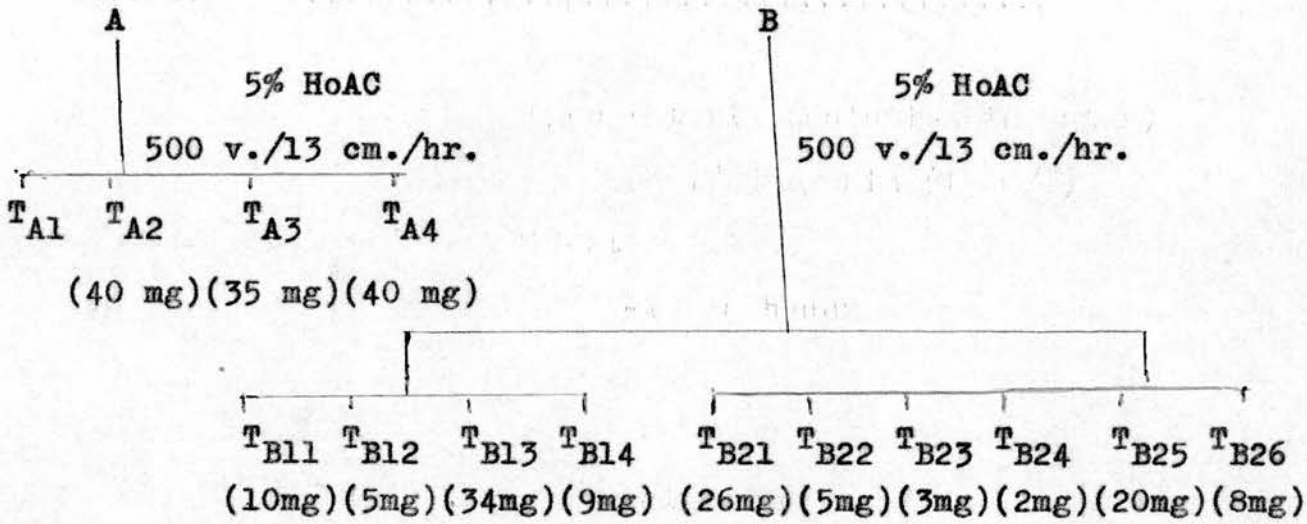


Table 15B. Scheme of Cellulose Chromatography of T_{A2}

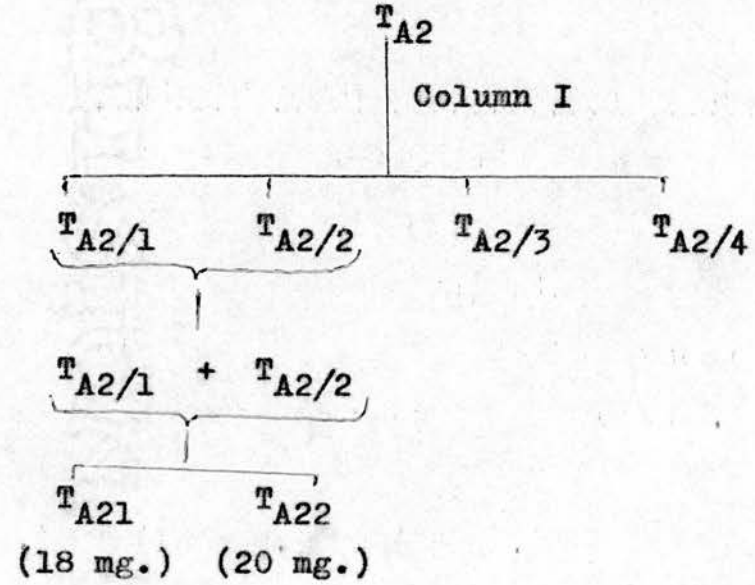


Table 16. Carbohydrate investigation of Bands T_{A1-4}, T_{B11-14}, T_{B21-26}

(Phenol/sulphuric acid estimation (after Dowex 50 W x 8)

Sample	Percentage carbohydrate
phycoerythrin	6.0%
T_{A1}	-
T_{A2}	7.0%
T_{A3}	5.0%
T_{A4}	5.0%
T_{B11}	3.7%
T_{B12}	3.1%
T_{B13}	4.0%
T_{B14}	3.6%
T_{B21}	3.5%
T_{B22}	3.0%
T_{B23}	3.0%
T_{B24}	3.0%
T_{B25}	4.0%
T_{B26}	3.5%

and were bulked to give the fractions shown in Table 15.A. The bulked fractions were freeze-dried and weighed.

Investigation of Electrophoretic Fractions

The electrophoretic fractions were first investigated by the chromatography-electrophoresis technique and this indicated how many components each band contained (G.P. 4). A sample of each fraction (1 mg.) was hydrolysed in a sealed tube (conc. H_2SO_4 for 18 hours) and the sample reduced in volume. The chromophore and amino-acid components of the hydrolysate were removed by adsorption on a short column of Dowex 50 WX8 (H^+) (8 cm. by 1 cm.). The resulting eluate was reduced in volume to 1 ml. and the phenol/sulphuric acid colorimetric technique was performed on this portion.

A series of standard estimations revealed that before reproducible results could be obtained the resin had to be very carefully prepared and regenerated and the columns during the operation had to be protected from direct sunlight. The resin was prepared by washing exhaustively with hydrochloric acid (2 N) after heating the resin for several hours with a solution of sodium hydroxide (2 N). The results of the phenol/sulphuric acid estimations are shown in Table 16.

The fraction which contained the highest proportion of carbohydrate appeared to be Band T_{A2}. Two-dimensional chromatography-electrophoresis (thin layer and paper) (G.P. 4 and 7), paper and thin layer electrophoresis (G.P. 3 and 6) and paper chromatography (G.P. 1, D) indicated that it contained

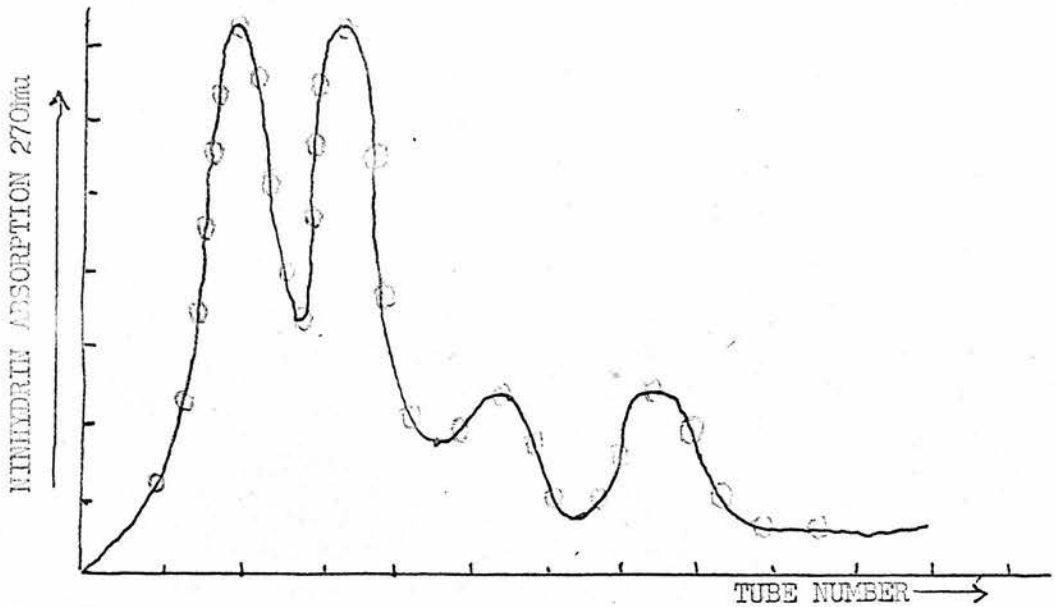


FIGURE 23 CELLULOSE CHROMATOGRAPHY OF BAND T_{A_2}

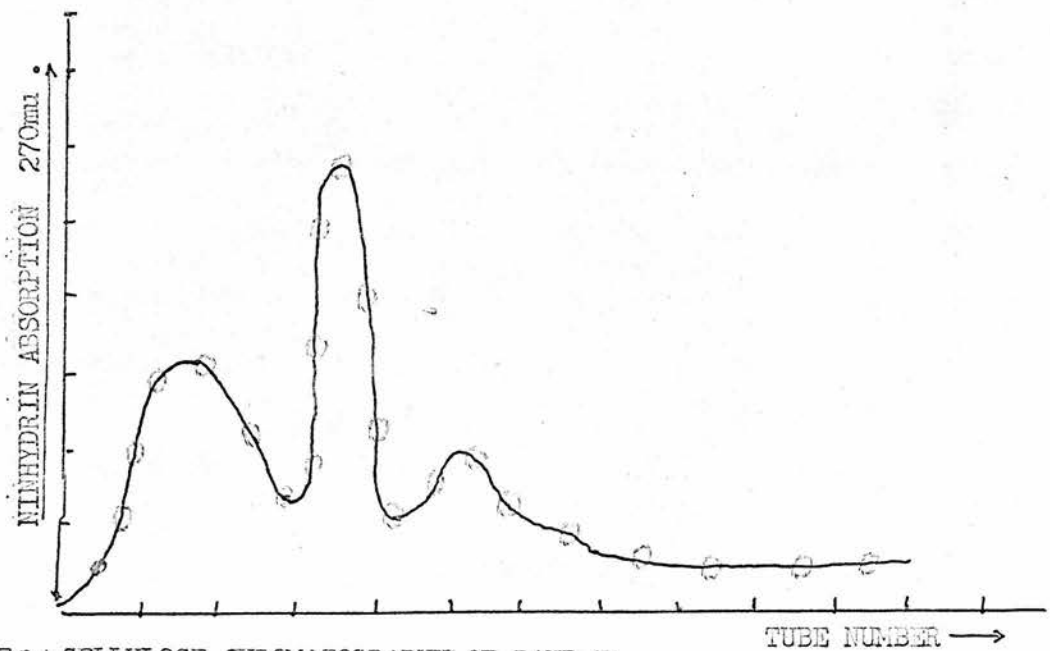


FIGURE 24 CELLULOSE CHROMATOGRAPHY OF BAND E .

four components. These components were of the same electrophoretic mobility but could be resolved chromatographically. A quantitative chromatographic procedure was developed from the qualitative paper procedure (Whatman No. 1, Solvent D). A column (22 cm. by 2 cm.) was filled with a slurry of cellulose in acetone and was packed carefully by slow displacement of acetone. The column was washed with several litres of acetone. The acetone was displaced by water by a stepwise elution with aqueous solutions of acetone and the column was washed with several litres of water. The water was replaced by a 50% aqueous solution of acetic acid and then finally butanol ; acetic acid : water (62:26:12). The column was finally washed with several litres of this solvent. Band T_{A2} was taken up in the solvent (10 ml.) and was applied to the column. The flow rate was adjusted to 6 ml. per hour and the eluate was collected in 6 ml. fractions. The eluate was examined by the solution ninhydrin procedure. A sample was withdrawn from every fifth tube, was reduced in volume to dryness, taken up in hydrochloric acid (2 ml. 6 N) and was hydrolysed (100°C, 18 hours). The hydrolysate was reduced in volume to dryness and the residue taken up on water (2 ml.). The samples were then submitted to the solution ninhydrin procedure (G.P. 18). A graph of ninhydrin positive material against tube number was constructed (Figure 23). From this it was deduced that a partial separation had been achieved.

The leading chromatographic band was bulked and re-chromatographed under the same conditions (flow rate 4 ml. per

Table 17. Amino-acid and sugar components in $T_{A2/1}$ and $T_{A2/2}$

Amino acids	$T_{A2/1}$	$T_{A2/2}$
aspartic	+++	++
glutamic	-	++
serine	+	-
threonine	-	+
glycine	+	+
alanine	++	++
arginine	+	+
methionine	-	+
valine	-	tr.
isoleucine	-	tr.
leucine	-	tr.
Carbohydrate		
galactose	-	-
glucose	+	+
arabinose	+	+
xylose	-	-
rhamnose	-	-
Percentage Carbohydrate	10-12%	13-15%
Methyl glycosides		
2,3,4,6-tetra-methyl glucose	++	++
3,4-di-methyl arabinose	+	+

hour). A second graph of the ninhydrin positive material against the tube number was constructed. The fractions were bulked to give Bands $T_{A2/1}$, $T_{A2/2}$, $T_{A2/3}$, $T_{A2/4}$. The solvent was removed by steam distillation and the fractions were taken up in water and freeze-dried (Table 16).

Fractions $T_{A1/1}$ and $T_{A1/2}$ were investigated by the usual electrophoretic and chromatographic techniques. Fraction $T_{A1/1}$ contained only one component which was homogeneous to electrophoresis and chromatography. Fraction $T_{A1/2}$ contained one major component and three minor components. The major component of Fraction $T_{A1/2}$ was isolated by quantitative paper chromatography (G.P. 1, D). These two fractions were then homogeneous to all methods of chromatography and electrophoresis so far employed and were regarded as pure one-component glycopeptides.

Investigation of the Homogeneous Glycopeptides

Samples of the two fractions (1 mg.) were hydrolysed (6 N HCl, 18 hours) and the amino-acid components were investigated (G.P. 2) (Table 17).

Samples of the two fractions (1 mg.) were resin hydrolysed at 100°C (G.P. 15) and the carbohydrate components in the water wash were investigated (G.P. 1, A). The results are shown in Table 17.

Samples of the two fractions (2 mg.) were hydrolysed (2 N H_2SO_4, 18 hours, 100°C) and the hydrolysates reduced in volume to dryness. The residues were submitted to the

phenol/sulphuric acid colorimetric procedure and the percentage carbohydrate in each fraction calculated (Table 17).

Samples of the two fractions (5 mg.) were methylated (G.P. 12) and methanolysed (G.P. 13). The methyl glycosides were investigated by gas-liquid chromatography (G.P. 11). The methyl glycosides isolated from the two fractions are shown in Table 17.

Investigation of Band E

Earlier investigation of Band E had indicated that although it was reasonably uniform and contained only two major components it was badly contaminated with salt and glycerol which had been released from the dialysis bagging. This glycerol and salt were removed from the main band by Sephadex chromatography. The sample was taken up in water and was chromatographed on a column of G-25 Sephadex (30 cm. x 1.8 cm., solvent water, G.P. 17). The column was eluted at a speed of 10 ml. per hour and 3 ml. fractions were collected. A sample from each tube (1 ml.) was tested for carbohydrate by the phenol/sulphuric acid method (G.P. 14, A) and also for amino-acid (G.P. 18). The ninhydrin positive band which was not grossly contaminated with carbohydrate was bulked and freeze-dried.

Investigation of Band E by the normal chromatographic and electrophoretic methods indicated that the band could be resolved into at least four components chromatographically (G.P. 1, D). The band was taken up in a few mls. of butanol : acetic acid : water and was chromatographed in exactly the same way as T_{A2} was on a similar cellulose column. A ninhydrin investigation procedure carried out on hydrolysed aliquots indicated that the cellulose chromatography had resulted in resolution of B and E into three main fractions (Figure 24).

Band 1 (30 mg.), Band 2 (62 mg.), and Band 3 (19 mg.).

Carbohydrate estimation by the phenol/sulphuric acid method (G.P. 14) indicated that the three bands contained less than 3% carbohydrate.

EXPERIMENTAL SECTION 3

Pronase hydrolysis

In her attempts to isolate a homogeneous chromopeptide from phycoerythrin Fujiwara[44] describes secondary digestion of a tryptic hydrolysate with the protease pronase p. Pronase p is a highly purified enzyme preparation extracted from Streptomyces griseus cultured broth. The enzyme is a high activity non-specific protease which is most effective working at pH 7.0 and at 55°C. Several authors have employed pronase in attempts to obtain glycopeptide and the experimental method described here was initially based on a method described by Marks *et al.*[55] for the digestion of egg albumin.

Initial small-scale experiments involving digestion of portions of phycoerythrin (20 mg.) with pronase (2% w/w) in calcium chloride solution (0.015 M, pH 8.5) under controlled conditions using a pH-stat indicated that reasonably effective cleavage resulted at 38°C. The digest was kept under nitrogen and the amount of acid released during proteolysis was recorded by the measurement of the alkali added (0.1 N, NaOH) to maintain constant pH.

Digestion with pronase was as expected fairly rigorous and reduced the protein to peptides which had an average M.W. of the order of 400-600.

A portion of phycoerythrin (200 mg.) was digested with pronase as described previously for 48 hours. The uptake of base was of the same order as that in the small-scale hydrolysis.

The digest was combined with the digests from the previous experiments and was reduced in volume to 10 ml. The solution was made 0.1 M with respect to acetic acid and was centrifuged (1,800 r.p.m., 0°C, 30 minutes). The minor insoluble portion was discarded. The soluble digest solution was then subjected to chromatography on a column of G-25 Sephadex (3 cm. x 35 cm.) equilibrated with acetic acid (0.1 M) at a flow rate of 0.5 ml./minute. The eluate was collected in 5 ml. fractions and the visible spectrum of each fraction was taken. The major coloured band was spectrally identical to the tryptic peptide showing a single absorption maximum at 510 mμ. The colourless eluate was examined by hydrolysis of samples (1 ml.) with 6 N hydrochloric acid followed by solution ninhydrin estimations. The fractions containing ninhydrin-positive material were neutralised with freshly distilled triethylamine and reduced in volume to dryness. A sample from each fraction was investigated by paper chromatography (G.P. 1, D) and the fractions were bulked to give Fractions A-F.

Fractions A-F were investigated by thin layer and paper electrophoresis-chromatography (G.P. 4 and 7). Development of the peptide maps with ninhydrin (G.P. 1, III) indicated that each band contained at least eight components and development with silver nitrate/sodium hydroxide (G.P. 1, II) and concentrated sulphuric acid (thin layer maps) indicated that several peptides contained sugar components. It was not possible to resolve the complex mixture in the limited time available.

Phycoerythrin (500 mg.) was dissolved in a solution of

calcium chloride as before and was incubated with pronase as described previously. The acid release was of the same order as before. The digest was reduced in volume and was centrifuged (3,600 r.p.m., 0°C, 15 minutes) and the soluble portion chromatographed in 0.1 M acetic acid on G-25 Sephadex. Examination of the eluate followed the same pattern as in the previous experiment and the digest was resolved into seven fractions: 1 (7 mg.), 2 (82 mg.), 3 (50 mg.), 4 (350 mg.), 5 (3 mg.), 6 (2 mg.), 7 (24 mg.), and an insoluble fraction (150 mg.). Band 4 proved to be highly contaminated with salt.

Small portions of the Bands 1-7 were investigated by the phenol/sulphuric acid colorimetric technique involving the Dowex 50 Wx8 ion exchange resin and it was discovered that all the bands contained ca. 4-6% carbohydrate. Investigation of the bands by paper and thin layer electrophoresis-chromatography (G.P. 4 and 7) indicated that the bands contained between five and seven components to a band.

Band 2 which comprised five components and which contained 4.5% carbohydrate was considered the most interesting for further study. The band was subjected to quantitative paper electrophoresis in the Wieland-Fischer apparatus on Whatman 3 MM paper at 450 v, 15 ma (22 v/cm.) in 5% acetic acid. Location of the separated bands was determined by development of contact prints of Whatman No. 1 paper. The electrophoresis resulted in fractionation of Band 2 into five main bands. Carbohydrate estimation, as described previously, revealed that all the bands contained roughly the same amount of carbohydrate

(i.e. 4-5%).

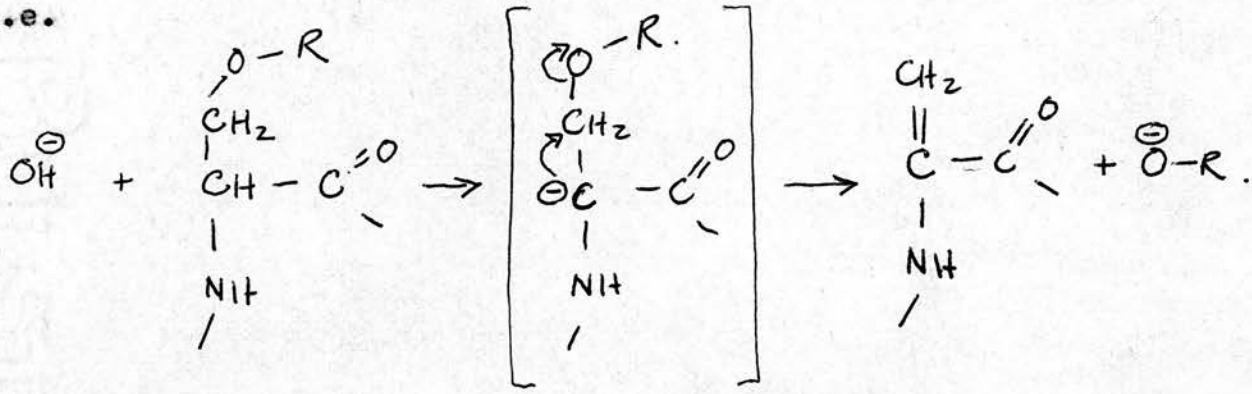
Further electrophoretic-chromatographic investigation of these bands under slightly more rigorous conditions (1½ hours electrophoresis, redeveloped chromatography) indicated that each of the bands contained at least three components. The peptide maps were developed with ninhydrin and with silver nitrate/sodium hydroxide (paper) or with ninhydrin and sulphuric acid (thin layer) and this revealed that all the peptides appeared to contain bound carbohydrate.

EXPERIMENTAL SECTION 4

Alkaline elimination and reduction

The amino-acid-carbohydrate linkage in chondroitin sulphate has long been known to be alkali-labile. It was proposed that this alkali-labile link was a carbohydrate ester involving the free carboxyl of aspartyl or glutamyl residues. Recent evidence has shown that 60% of the serine present in chondroitin sulphate disappears on alkaline hydrolysis and this suggests that this serine is involved in a serine-carbohydrate glycosidic bond. Such a glycosidic linkage would be liable to elimination with resultant destruction of serine,

i.e.



as described by Anderson, Hoffman and Meyer.[104] A similar O-glycosidic linkage is also probable involving threonine.

β-carbonyl elimination of the type described would yield dehydroalanine and 2-amino crotonic acid from the serine and threonine involved. These unsaturated amino-acids proved hard to isolate from hydrolysates after alkaline and acid hydrolysis and for some time the relative proportions of serine and

threonine before and after alkaline hydrolysis were the sources of evidence for this O-glycosidic linkage.

However, Tanaka, Bertolini and Pigman[51] have shown that alkaline treatment of O.S.M. accompanied by reduction with sodium borohydride resulted in a loss of serine and threonine accompanied by the formation of alanine and α-amino butyric acid. This means of stabilizing and identifying the products of alkaline hydrolysis has been employed and developed by several workers.

The procedure normally employed is to digest a solution of glycoprotein at low temperature with an aqueous alkaline solution of sodium borohydride. After removal of salt by dialysis the protein moiety is subjected to normal amino-acid analysis.

The double bond of 2-amino crotonic acid has proved resistant to reduction and an improvement has been suggested by Tanaka and Pigman[105] using palladium chloride as a catalyst.

Experiment 1

A sample of phycoerythrin (50 mg.) was dissolved in distilled water (50 ml.); the solution was centrifuged (4,800 r.p.m., 30 min., 0°C) and was dialysed against distilled water (0°C, 24 hrs.). The pH was adjusted to pH 10.4 with a solution of sodium hydroxide (0.1 M) and the solution was heated at 80°C for 24 hours under nitrogen. After 24 hours the pH had dropped to pH 9.1 indicating a release of acid. The solution was neutralised with acetic acid (0.1 N) and was

dialysed against distilled water for 24 hours. The dialysate was reduced in volume (final volume, 20 ml.) and was deionised (IR 120, IR 45). The deionised solution was evaporated to a syrup. The non-dialysable fraction was freeze-dried.

A sample of the dialysate was examined by paper chromatography for carbohydrate components (G.P. 1, A,B,C) and traces of glucose and galactose could be located. It was suspected that most of the carbohydrate had been converted into saccharinic acids. The non-dialysable fraction was split into two parts. One sample (10 mg.) was hydrolysed (6 N HCl, 48 hours) and the amino acid constituents examined by paper chromatography (G.P. 2). The amino acid pattern was identical to that obtained by hydrolysis of phycoerythrin. The spots which corresponded to alanine, serine, threonine and tyrosine were cut from the chromatogram and from a standard phycoerythrin chromatogram. The spots were each washed with ethanol diluent (50/50 ethanol/water, 2 ml.) and the wash was examined at 570 mμ for ninhydrin absorption. No great variation in the ratio of serine:alanine, threonine:alanine, serine:tyrosine, threonine:tyrosine could be observed. The second portion of the non-dialysable fraction was resin hydrolysed (100°C) and the neutral wash was examined for carbohydrate constituents (G.P. 1, A,B,C). Glucose, galactose, arabinose and xylose were detected indicating the presence of residual bound carbohydrate after alkaline hydrolysis.

Experiment 2

A portion of phycoerythrin (100 mg.) was dissolved in tris buffer (pH 8.0, 0.1 M, 100 ml.). The solution was centrifuged (3,000 r.p.m., 15 min., 0°C) and sodium borohydride (50 mg.) was added to the solution. A few drops of octyl alcohol were added to prevent frothing. The solution was incubated at 70°C under nitrogen for six hours. The solution was then neutralised (0.1 M acetic acid) and dialysed against distilled water (24 hrs.). The dialysate was reduced in volume (final volume, 10 ml.) and was deionised (IR 120, IR 45). The deionised solution was evaporated to a syrup. A sample was examined by paper chromatography (G.P. 1, A,B) for carbohydrate constituents and two components which appeared to be glucose and galactose were located. To determine whether these sugars were the free reducing sugars or the reduced sugar alcohols the dialysate was investigated by means of a solvent which splits alcohols and reducing sugars, i.e. methyl ethyl ketone, acetic acid, water (saturated with boric acid), (9; 1:1). The chromatograms were sprayed with sodium periodate-potassium permanganate-benzidene spray reagent.[106] It was established that the carbohydrate present was free non-reduced glucose and galactose. The non-dialysable fraction was free non-reduced glucose and galactose. The dialysable fraction was freeze-dried and a sample of the freeze-dried protein (5 mg.) was hydrolysed (G.P. 9, B). The amino acids in the hydrolysate were examined by paper chromatography (G.P. 2). The amino-acid composition of this treated protein was identical to that

of native phycoerythrin. A further sample (10 mg.) was resin-hydrolysed (G.P. 13) and the carbohydrate constituents of the water wash were identified by paper chromatography (G.P. 1, A, B). It was observed that glucose, galactose, arabinose and xylose were still bound to the protein after this treatment.

Experiment 3

Phycoerythrin (100 mg.) was dissolved in 15 ml. of 0.3 M sodium borohydride in 0.1 N sodium hydroxide. A few drops of octanol were added to prevent frothing. The solution was stirred for 72 hours at 0°C. The sodium borohydride was destroyed with acetic acid (1 N) and the resultant solution (pH 5.3) was dialysed against distilled water for 24 hours. The dialysate was reduced in volume (final volume, 10 ml.) and was passed through a column of IR 120 to remove sodium ions. The boric acid was removed under vacuum as methyl borate. The resultant syrup was further deionised by passage through columns of IR 120 and IR 45 and was evaporated to a syrup. This deionised dialysate was investigated for free carbohydrate components by chromatography. Samples of the dialysate were run in methy ethyl ketone; acetic acid : water (9; 1:1) and the carbohydrate located by sodium periodate-potassium permanganate-benzidene in addition to the normal spray reagents. Results indicated the presence of carbohydrate (confirmed by phenol/sulphuric acid colorimetry) but resolution in this and other solvents proved difficult because of residual ionic contamination. It had been observed during the course of the

hydrolysis-reduction that a yellow coloured artifact which exhibited green fluorescence was released and entered the octanol layer. This artifact was removed by extracting the non-dialysable fraction with butanol. The fraction was then freeze-dried. A sample was hydrolysed (6 N HCl for 48 hours; G.P. 9, B) and the amino-acid pattern was very similar to that of phycoerythrin. The amino-acids were also submitted to chromatography in n-propanol : water (80:20) on Whatman No. 4 chromatography paper. The hydrolysate was observed to contain traces of α-amino butyric acid which indicated that reduction of threonine had taken place. The spots corresponding to alanine, glycine and serine, threonine and α-amino butyric acid were cut out and their relative ninhydrin absorptions at 570 mμ in ethanol-water (50-50, 3 ml.) were taken. These absorptions were compared to these determined for hydrolysed phycoerythrin and results indicated that threonine had been reduced to α-amino butyric acid but that there was no change in the relative serine proportions.

Experiment 4

In order to obtain slightly more conclusive evidence of the involvement of serine and threonine in secondary carbohydrate bonding using alkaline elimination coupled with reduction, experiments involving sodium borotritilide were carried out. It could be expected that the use of sodium borotritilide in the reduction stage would result in incorporation of tritium in the resultant reduced amino-acids and

carbohydrate. Reduction of the products of β-carbonyl elimination of serine and threonine would result in alanine and α-amino butyric acids which had each incorporated two atoms of tritium.

Theoretical assessment:

100 μ of sodium borotritilide were diluted with 0.48 g. of sodium borohydride

$\therefore$ Dilution factor = 10^{-4} g. in 0.48 g.

= 2×10^{-4} . . . . . (1)

Serine in phycoerythrin = 10%

Estimated involvement in glycosidic bond = 10%

Weight of protein used = 50×10^{-3} g.

$\therefore$ Weight of serine involved in bond = 5×10^{-4} g. . (2)

M.W. of serine = 105

$\therefore$ No. of serine units involved (from (2)) = 5×10^{-6}

$\therefore$ No. of tritium atoms involved (i.e. 2 per molecule)

= 10^{-5} . . . . . (3)

$\therefore$ No. of tritium atoms after dilution ((1) and (3))

= 2×10^{-9}

Paper chromatography of 5 mg.

$\therefore$ No. of tritium atoms chromatographed = 2×10^{-10} . (4)

Tritium count = 722 mc/mm

= 180 mc/m. atom

= 180×10^{3} mc/atom . . . . (5)

$\therefore$ Count of incorporated tritium = $180 \times 10^{3} \times 2 \times 10^{-10}$ mc.

= <u>36 anocuries</u>

This assessment predicted that the uptake would be measureable using the detector available, i.e. the Packard Tricarb Liquid Scintillation Counter Model 314 ex. The samples were counted in polythene vials in scintillation solution A.

Scintillation solution A:

napthalene (88 g.)

2,5-diphenyloxazole (8.6 g.)

1,4-bis-2(4-methyl-5-phenyloxazole-benzene) (70 mg.)

xylene (200 ml.)

dioxane (1,000 ml.)

0.5 ml. H_2O/15 ml. solution

The procedure used in Experiment 3 was repeated and immediately after addition of the phycoerythrin to the sodium hydroxide solution an addition of 100 μ of sodium borotritilide was made. The experiment was carried out in a radioactive laboratory and the normal precautions for β-emitters were taken. The sodium borotritilide was obtained from the Radiochemical Centre, Amersham, Bucks.

The experiment was carried out in a reaction vessel cooled to 0°C. After 72 hours a 5 ml. sample of the digest was withdrawn. After dilution to 20 ml. the sample was neutralised with acetic acid (0.1 N) then dialysed against distilled water. The non-dialysable fraction was freeze-dried and the freeze-dried sample hydrolysed (6 N HCl, 48 hours; G.P. 9, B). The amino acids were chromatographed in propanol:water (80:20) on Whatman No. 4 (18 hours). The hydrolysate was observed to

contain α-amino butyric acid. The spots corresponding to serine, glycine, threonine, alanine, aspartic acid and α-amino butyric acid were carefully cut from the paper (using a circular cutter). These paper circles were placed in the polythene counting vials and were covered by equal volumes of counting fluid. A count of the tubes over a five-hour period was made.

The chromatography and counting procedure were duplicated. On the first occasion the amino-acids were developed by ninhydrin and on the second their positions relative to standards were estimated and the untreated spots cut out. The count gave further proof of the involvement of threonine in a secondary ether linkage.

Table 18. Results of tritium count.

Ninhydrin spots		Calculated position spots	
Amino-acid	Count	Amino-acid	Count
Blank	310	Blank	289
alanine	291	alanine	301
α-amino butyric	575	α-amino butyric	597
threonine	382	threonine	322
serine	313	serine	308

The chromatography was repeated and the chromatogram was developed with ninhydrin. The continuous line of the amino acid spots was cut out from the chromatogram and the count of each spot taken. The result once more indicated that α-amino butyric acid was the only one with incorporated tritium. A

graph displaying count against amino-acid chromatographic placement emphasises this result (Figure 25).

EXPERIMENTAL SECTION 5

Carbohydrase-phycoerythrin digestion

For several years general and specific carbohydrases have been used in the elucidation of the structures of polysaccharides. Recently, this technique of hydrolysis of glycosidic bonds with enzymes has been applied to structural studies of glycoproteins. Montgomery[107] isolated a carbohydrate-rich glycopeptide from ovalbumin and has shown that its constituents are:

D-mannose (3 molecular proportions),

N-acetyl D-glucosamine (3 molecular proportions),

and aspartic acid (1 molecular proportion).

Hydrolysis of the fragment with almond emulsin before and after dinitrophenylation has indicated that the central glycopeptide involves aspartic acid and the reducing group of N-acetyl glucosamine. Eylar[108] has succeeded in isolating an enzyme from sheep epididymis which catalyses the hydrolysis of the β-aspartylglycosylamine linkage. This enzyme has provided a further means of establishing the 1-β-aspartyl-2-acetamido-1,2-dideoxy-D-glucosylamine structure presented for the α-1-glycoprotein. Extensions of this work could provide an armoury of specific enzymes which preferentially cleave carbohydrate-protein bonds.

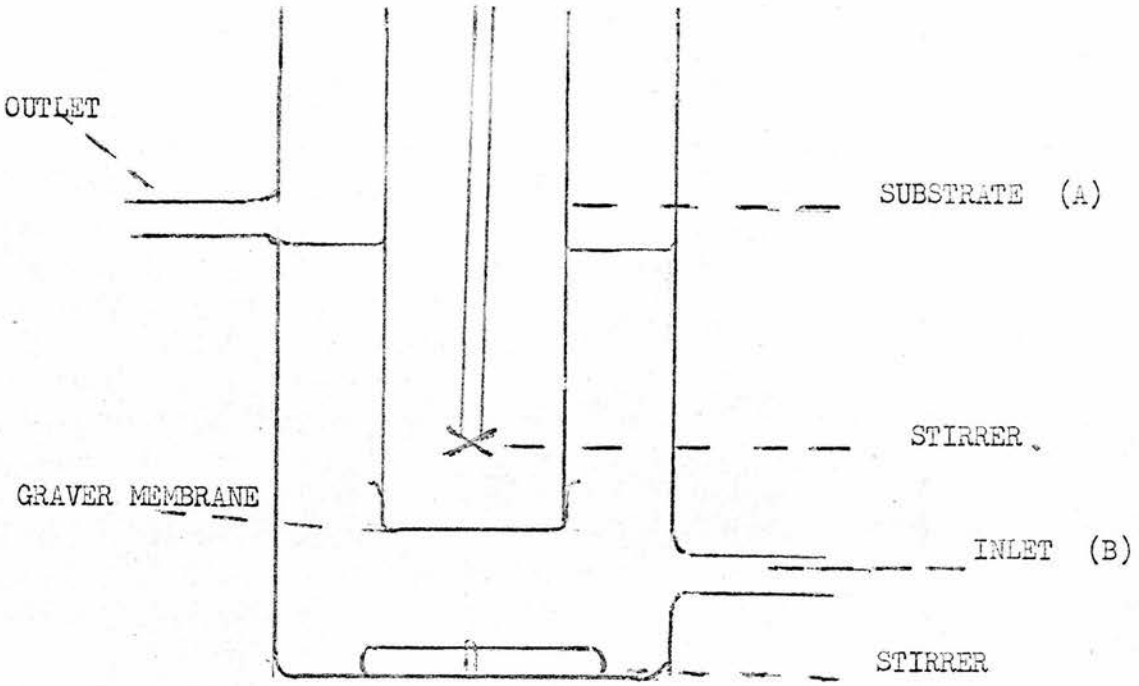
Hemicellulases are non-specific carbohydrases which are present in all branches of nature, i.e. plants, fungi, bacteria, etc. They are most active in the region of

pH 4.0 - 7.0 and at temperatures of up to 60°C. They catalyse hydrolysis of glycosidic bonds between all the common carbohydrate monomers. It was decided to digest a sample of phycoerythrin with fungal hemicellulase and investigate the products of the digestion.

Experimental

The activity of commercial fungal hemicellulase (Light and Co.) was tested. Samples (5 mg.) of laminarin, a mannan, a galactomannan and a larch xylan were digested at 50°C for 18 hours in citrate/phosphate buffer (0.01 M, pH 6.0) (enzyme/substrate, 1/50). After digestion the samples were reduced in volume to a syrup and the carbohydrate investigated by chromatography (G.P. 1, A). The results indicated that the enzyme was completely non-specific and resulted in release of all the monomer units involved in the samples. It could be expected on this basis that all the carbohydrates involved in phycoerythrin would be released provided they are linked to the protein moiety through some normal carbohydrate linkage. Phycoerythrin (100 mg.) was dissolved in phosphate/citrate buffer (100 ml., 0.01 M, pH 6.0) and hemicellulase in aqueous solution was added (1 mg. in 1 ml.). The digest was incubated in a sterilised flask at 50°C for 18 hours. The digest was filtered and the remaining insoluble material thoroughly washed. The soluble portion and washings were reduced in volume to a final volume of 5 ml. This sample was chromatographed on G-25 Sephadex (column 1.2 cm. x 12 cm.). The eluate was collected

in 3 ml. fractions using a fraction collector. The fractions were investigated for the presence of carbohydrate (phenol/ sulphuric acid colorimetry G.P.) and the results indicated that the carbohydrate was located in the same band as the coloured protein moiety. Rechromatography failed to separate the two. The coloured eluate was then dialysed against distilled water using a microdialysis technique. Distilled water was circulated through a dialysis cell by a micro pump at a rate of 10 ml./hour. The dialysate was collected and was reduced in volume to a syrup. This dialysate contained carbohydrate (phenol/sulphuric acid colorimetry) which was shown by paper chromatography to be glucose, galactose, arabinose and some traces of oligosaccharide material (G.P. 1, A and B). The non-dialysable material was freeze-dried and a sample of this freeze-dried fraction was resin-hydrolysed at 100°C (G.P. 14). The water wash was found to contain traces of glucose, arabinose and xylose. Estimation of the carbohydrate content of this freeze-dried sample was carried out by direct phenol/ sulphuric acid colorimetry and the results indicated that over 60% of the carbohydrate constituents had been cleaved from the protein moiety by the hemicellulase. The validity of the experiment was confirmed by the digestion of a hemicellulase blank and a hemicellulase/Sephadex blank. The investigation of the products by paper chromatography (G.P. 1, A) indicated that the carbohydrates isolated in the previous procedure were not artifacts. The experiment was repeated with exactly the same results.



(A) SUBSTRATE- HEMICELLULASE AND PHYCOERYTHRIN IN AQUEOUS SOLUTION .

(B) DISTILLED WATER HEATED TO 38°C USING A CIRCOTHERM HEATING UNIT PUMPED BY A MICROPUMP AT 10ml/hour .

FIGURE 26 ENZYMIC DIALYSIS UNIT

The presence of traces of oligosaccharide components in the hemicellulase/phycoerythrin digest dialysate was considered interesting and was in agreement with results from the investigation of the resin hydrolysate of a tryptic peptide which indicated the presence of similar oligosaccharides. The quantities of oligosaccharides obtained by hemicellulase digestion prevented any reasonable investigation. However, Painter[109] has evolved a method whereby, by digestion of polysaccharides with carbohydrase preparations in a dialysis bag surrounded by water at the required incubation temperature, the oligosaccharide/monosaccharide ratio was much higher than that obtained by digestion with a carbohydrase followed by dialysis. The idea is that the oligosaccharides originally cleaved from the polysaccharide can dialyse into enzyme-free water before further hydrolysis and formation of monomers can take place. Painter was successful in obtaining greatly increased yields of oligosaccharide by this method. Similar experiments involving partial hydrolysis of polysaccharides with polystyrenesulphonic acid are also described. Bishop[110] described an enzymic isolation of oligosaccharides from a glucomannan from jack-pine using the Painter dialysis technique. He describes a dialysis cell upon which the dialysis cell described in Figure 26 was based. The following experiments were performed in this cell.

1) Several preliminary blank trials were carried out using the enzymic dialysis cell in order to test the dialysis membrane against hemicellulase action. It was discovered that digestion of the Visking tubing with hemicellulase resulted in

a rapid release of glucose and that within a few hours the dialysis membrane collapsed. Tests carried out using the Graver polyvinylchloride dialysis membrane indicated that the dialysate was free from carbohydrate and that the membrane was resistant to enzymic action and to temperatures up to 60°C. The membrane was resistant to collapse even when exposed to digestion at high temperatures for long periods.

2) A sample of phycoerythrin (15 mg.) was digested with hemicellulase (enzyme/substrate, 1/20) in aqueous solution in the dialysis cell at 38°C. The dialysate was collected in 20 ml. fractions. The fractions were examined for carbohydrate by phenol/sulphuric acid colorimetry and the carbohydrate-rich fractions were investigated, after reducing in volume to syrup, by paper chromatography (G.P. 1, A). The dialysate collected over the first six hours was found to contain two major components which moved at half the rate of galactose in Solvent 1, along with equal quantities of the usual monomer units, galactose, glucose and arabinose. The latter portions of the dialysate contained only the monomer carbohydrates.

3) The experiment was repeated and a further sample of phycoerythrin (50 mg.) was digested with hemicellulase under exactly the same conditions. The dialysate was divided into two main fractions A, (0-2 hrs.); B, (0-8 hrs.) and was freeze-dried. The non-dialysable material was also freeze-dried. Resin hydrolysis of a sample (10 mg.) of the non-dialysable fraction and subsequent investigation of the water

and acid washes indicated that this fraction was mainly protein or peptide containing the amino acids known to be present in phycoerythrin. Traces of the carbohydrate monomers were located. Fraction A was found to contain what appeared to be oligosaccharide material (G.P. 1, A; R_g 0.5). Hydrolysis of a sample of Fraction A (G.P. 8, B) followed by investigation of the products revealed that the oligosaccharide material disappeared on hydrolysis. Incubation of a sample of Fraction A with hemicellulase in aqueous solution resulted in the disappearance of oligosaccharides. Hydrolysis of a sample of Fraction A (G.P. 8, A) and investigation of a portion of the hydrolysed and native Fraction A by ninhydrin colorimetry (G.P. 18) indicated that the fraction contained no protein or peptide material.

4) A further experiment was carried out using denatured phycoerythrin. A sample of phycoerythrin (100 mg.) dissolved in distilled water (100 ml.) was stirred at 38°C in the enzymic dialysis cell until the native phycoerythrin fluorescence was quenched. The dialysate (A) was collected. Hemicellulase (5 mg.) was added to the denatured phycoerythrin solution and the enzymic dialysis continued for six hours. The dialysate (B) was reduced in volume (final volume, 50 ml.) and was freeze-dried. The non-dialysable material was freeze-dried. Investigation of Fractions A, B and C by hydrolysis (G.P. 8, B) followed by paper chromatography (G.P. 1, A) and by paper chromatography (G.P. 1, A) on the native fraction and on the native fractions after hydrolysis (G.P. 8, A) and digestion

with hemicellulase was carried out.

The results are given in Table 19.

Table 19. Enzymic Dialysis Results.

	Protein and peptide	Free carbohydrate	Bound carbohydrate
Band A	-	-	-
Band B	-	oligosaccharides (+) glucose (++) galactose (+) arabinose (+)	-
Band C	All amino-acid components of phycoerythrin	-	glucose (++) galactose (+) arabinose (+) rhamnose (+)

5) The enzymic dialysis procedure was also repeated with the pH-stat and recorder (Radiometer, Copenhagen) measuring the release of acid. The extent of proteolysis was so small that the release of acid could not be recorded.

Several attempts to separate the oligo- and monosaccharides of Band B were made in an effort to characterise these oligosaccharides. Band B was submitted to thick paper quantitative chromatography. Band B was dissolved in water (1 ml.) and was spotted along the marked baseline on a sheet of Whatman 3 MM paper. The chromatogram was developed for 20 hours (Solvent 1, A). The positions of sugars after separation was ascertained by cutting out and developing (G.P. 1, y) narrow centre and side stripes. The sugars were eluted from the paper with cold water. The quantities of carbohydrate in

Band B were so small that the contamination from arabinose washed from the paper rendered the fractions obtained from quantitative chromatography useless. Pre-washing papers did not inhibit this release of arabinose. Band B was submitted to chromatography on a column of Dowex 50wx 2 (Li^+) (50 cm. x 0.8 cm.) and the components were to some extent resolved.[111] Attempts were made to methylate and methanolyse (G.P. 12 and 13) the oligosaccharide fraction and to identify the methyl glycosides by gas liquid chromatography (G.P. 11) but the small amounts involved could not be resolved properly and only trace quantities of 2,3,4,6-tetra-O-methyl glucose could be picked up by the detector. Several attempts to scale up the enzymic dialysis and subsequent separation, methylation and methanolysis procedure did not yield conclusive results.

D I S C U S S I O N

The extraction and fractionation of the biliproteins from Rhodymenia palmata did not present any real problems. The method described in the experimental section resulted in a reasonably fruitful extraction and a clear cut fractionation. The visible spectral patterns of the extracted biliproteins appeared to be very similar to the spectra of the R-phycoerythrins and R-phycocyanins as described by other workers. The ratio of the λ max at 479 mμ to that at 275 mμ is used as a criterion of purity by investigators of the B-phycoerythrins and the value of 2.5 obtained for the extracted phycoerythrin seems to indicate that it is reasonably pure. Attempts to crystallise the extracted phycoerythrin were not successful and it is suspected that this was due to the slight contamination with rivanol which was involved in the fractionation procedure and which was very difficult to remove completely. It is, however, accepted that the phycoerythrin from Rhodymenia is difficult to crystallise (O'Carra, personal communication).

Investigation of the amino-acid components of the extracted phycoerythrin revealed that it contained the same amino acids in approximately the same relative proportions as those generally accepted for the phycoerythrins. There did not appear to be any seasonal differences in the amino-acid pattern nor in the visible absorption spectrum. The chromophore was hydrolysed and extracted by the classical Lemberg technique and its visible spectrum in acid chloroform was examined - this proved to be identical to that of the extracted, modified phycoerythrobilin obtained by Lemberg.

Investigation of the hydrolysate of the extracted phycoerythrin after a series of precipitational and chromatographic procedures revealed that the phycoerythrin contained bound carbohydrate. Further experiments involving proteolytic hydrolysis, chromatography and electrophoresis further emphasised that this carbohydrate must be chemically bound and not a contaminant or artifact.

Fujiwara[44] and Tsuchiya[46] have investigated the carbohydrate components of the phycoerythrin from Porphyra tenera and their results are shown below.

	galactose	glucose	arabinose	mannose	xylose	rhamnose	amino-sugar
Fujiwara	+		+		+	+	+
Tsuchiya	+	+		+	+	+	

Fujiwara also described the isolation of some slower moving chromatographic material which she identified as a galactose-containing disaccharide.

Careful chromatographic examination, using a series of solvent systems, of the carbohydrate components of the complete acid and resin hydrolysates of the phycoerythrin from Rhodymenia shows that they are: galactose, glucose, arabinose, xylose and rhamnose. No traces of amino-sugar, as reported by Fujiwara, nor any sugar sulphate, as predicted in terms of the sulphur content of the original protein, could be located. These results agree almost completely with those of Tsuchiya. The mannose he reports could in fact be arabinose since the solvent

he uses would not separate arabinose and mannose. The amino sugars reported by Fujiwara could not be located. The solvent she uses would not differentiate galactose and glucose which explains the absence of glucose in her results.

On the basis of the more positive evidence from the work of Fujiwara and Tsuchiya and this present study we can deduce that the monomer carbohydrate units bound to phycoerythrins from Porphyra tenera and Rhodymenia palmata are the five mentioned above.

A series of attempts to estimate the amount of bound carbohydrate present in phycoerythrin were carried out using the standard colorimetric techniques. The amount of carbohydrate bound to phycoerythrin was calculated as 5%, which agrees closely with the figure quoted by Fujiwara (4.78 - 4.99%). One rather unusual feature observed was that on hydrolysis of the protein before carbohydrate estimation the percentage of reducing carbohydrate rose to 7%. This may be explained in terms of the protein-carbohydrate linkage and it seems likely that this 30% increase in the reducing power may be due to the cleavage of glycosylaminyl linkages between glutamine and asparagine residues and the reducing group of a carbohydrate molecule. This linkage would not be cleaved under the non-aqueous conditions of the phenol/sulphuric acid method. This same effect has been reported by Marks et al.[112] as a feature of ovalbumin where it has been proved conclusively that the major type of linkage is glycosylaminyl.

The hexose:pentose ratio obtained by the p-aminobenzoic

acid method is approximately 2:1 and this agrees with the paper chromatographic evidence. This was confirmed by quantitative paper chromatography and the relative carbohydrate monomer proportions were estimated as: glucose (4), galactose (2), arabinose (2), xylose (1) and rhamnose (trace).

From the result that phycoerythrin contains 7% carbohydrate and the known M.W.s of the protein (300,000) and a typical carbohydrate unit (176) it can be calculated that there must be 120 carbohydrate units per molecule or approximately 8 carbohydrate units per phycoerythrin subunit (assuming 16 subunits per molecule).

In certain resin-hydrolysates of phycoerythrin reducing sugar could be detected which moved more slowly than the normal monomer sugars in basic solvents. This was thought to be a glucose-containing disaccharide, similar in chromatographic behaviour to what Fujiwara described as a galactose-containing disaccharide. Resin hydrolysis at lower temperatures resulted in an expected increase in the proportion of this oligosaccharide material. Further acid hydrolysis of the mild resin hydrolysate resulted in disappearance of the oligosaccharide material with subsequent increase in the amount of glucose present. Digestion of the mild resin hydrolysate with almond emulsin also resulted in release of glucose and disappearance of the oligosaccharide while digestion with maltase had no effect. Several attempts to isolate and further characterise this oligosaccharide from the results of methylation failed. On the basis of the evidence available we can

tentatively label this oligosaccharide as a β-linked glucose-containing disaccharide, probably cellobiose.

Initial small-scale proteolyses of phycoerythrin with trypsin were moderately successful. In these pilot experiments the release of acid was followed during the first few hours of the digestion and from this it appeared that a reasonable amount of proteolysis was taking place. The changes in the visible absorption spectra during proteolysis and the spectra of the bands obtained on Sephadex chromatography paralleled those described in previous workers' reports - degradation was accompanied by disappearance of the visible absorption at 520 mμ. Investigation of the largest molecular weight fraction after chromatography indicated that it was unchanged phycoerythrin. Redigestion of this fraction revealed that it was not trypsin-resistant protein but that it must have resulted from incomplete proteolysis.

Further fractionation was achieved by submitting the main bands to continuous electrophoresis. The groups of peptides thus obtained were examined and a rough idea of their composition was obtained. Investigation of their amino-acid components revealed that no fraction was simple enough to make a rigorous investigation of the carbohydrate-protein linkage and the small quantities of the fractions precluded the idea of further fractionation. Investigation of the carbohydrate components of the fractions indicated that one fraction was rich in a similar oligosaccharide to that obtained by mild resin hydrolysis. The fraction contained glucose, galactose

and arabinose in addition to this slower moving spot. Further investigation proved that it was a β-linked disaccharide which was chromatographically similar to cellobiose, i.e. the results indicated that it was identical to the oligosaccharide obtained by mild resin hydrolysis. Ion exchange chromatography proved that the carbohydrate was bound to the protein moiety in a glycopeptide structure. The complexity of the amino-acid pattern forbade any conclusion regarding the nature of the bond save that it was unlikely that the chromophore was involved in the linkage to the carbohydrate since the visible absorption spectrum of the fraction revealed no involvement of the chromophore.

Proteolysis on a very much larger scale was carried out in an attempt to isolate a homogeneous glycopeptide in which the amino-acid composition was simple enough to make accurate structural observations regarding the carbohydrate-protein linkage. This large-scale proteolysis was carried out under controlled conditions using a pH-stat and resulted in a complete degradation of the phycoerythrin in 48 hours with two additions of enzyme. The spectral pattern of the products of the reaction after Sephadex fractionation followed that of the pilot experiments. The fractions obtained by Sephadex chromatography were submitted to continuous electrophoresis. The band obtained from this second fractionation which was richest in carbohydrate was closely investigated. It was decided that the best means of further resolution would be by cellulose chromatography. After a rather complicated procedure

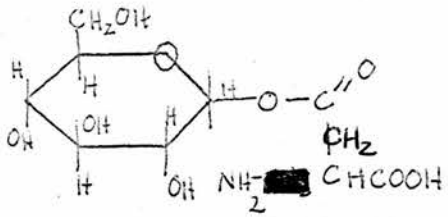
involving column and quantitative paper chromatography two carbohydrate-rich glycopeptides were isolated.

Rigorous investigation of the glycopeptides showed that the first was homogeneous to electrophoresis and chromatography but that the second was contaminated by several glycopeptides minor components.

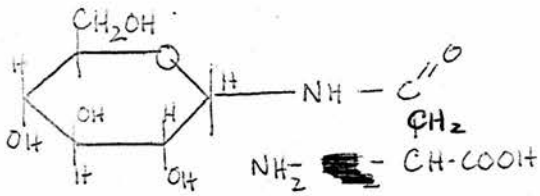
The amino-acid composition of the first of these glycopeptides was relatively simple and involved only aspartic acid, serine, glycine, alanine and arginine. Its carbohydrate components were glucose and arabinose which made up 10-12% of the glycopeptide. Methylation of the intact glycopeptide and investigation of the methanolysed products revealed that the glucose must be involved in a linkage through its reducing end and that the arabinose must be linked to the peptide through its reducing end or through the 2-position. The arabinose and the glucose must be present in their pyranoside forms.

From these results we can deduce that the glucose must be linked as a carbohydrate ester to aspartic acid (I) or through a glycosylaminyl link to asparagine (II). The possibility also exists for a glycosidic ether bond between glucose and serine (III). Linkage to arginine, although possible, seems unlikely. If the arabinose is linked through its reducing end with the 2-position involved in branching the same linkages (I, II and III) are possible. However, if the arabinose is bound via its 2-position the glycosidic bond is no longer possible and the (I) and (II) structures modify to give (IV) and (V).

The second glycopeptide contained 13-15% carbohydrate



(I)



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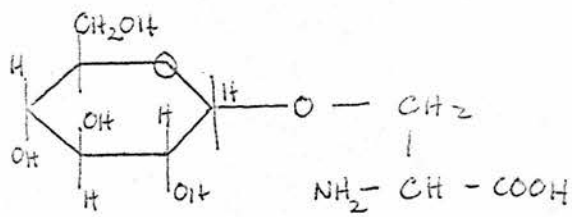
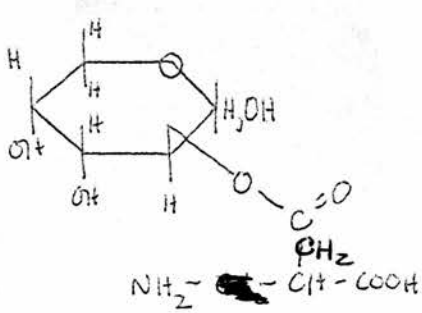
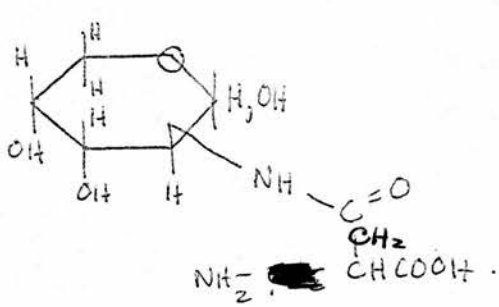


(III)



(IV)



(V)

FIGURE 27 AMINO-ACID - CARBOHYDRATE LINKAGES

which was totally glucose and arabinose. The amino-acid pattern was slightly more complicated and the peptide contained aspartic acid, glutamic acid, threonine, glycine, arginine, and methionine with traces of valine, isoleucine and leucine. Methylation and methanolysis of the intact glycopeptide showed that the glucose must be bound through its reducing end and that the pyranoside form of arabinose could be bound through its reducing end or through the 2-position. The glucose can either be linked as a carbohydrate ester to aspartic acid as in the case of the homogeneous glycopeptide (I) or similarly to glutamic acid. Asparagine and glutamine may be involved in glycosylaminyl linkages to glucose. There also exists the possibility of a glycosidic bond between glucose and theonine (III). All the types of bond described as possible for arabinose for the previous glycopeptide are also feasible for this second in addition to the possibility of the involvement of glutamic acid or glutamine in the same way as aspartic acid and asparagine are involved.

Tryptic hydrolysis followed by this eventual isolation of carbohydrate-rich glycopeptides as well as enabling us to have a clearer view of structural glycopeptide linkage has indicated that most of the carbohydrate must be involved in linkages with amino acids and not with the tetrapyrrole chromophore since no evidence of the chromophoric involvement could be detected in these carbohydrate-rich glycopeptides (for I, II, III, IV and V see Figure 27).

Proteolysis of phycoerythrin with pronase resulted in a

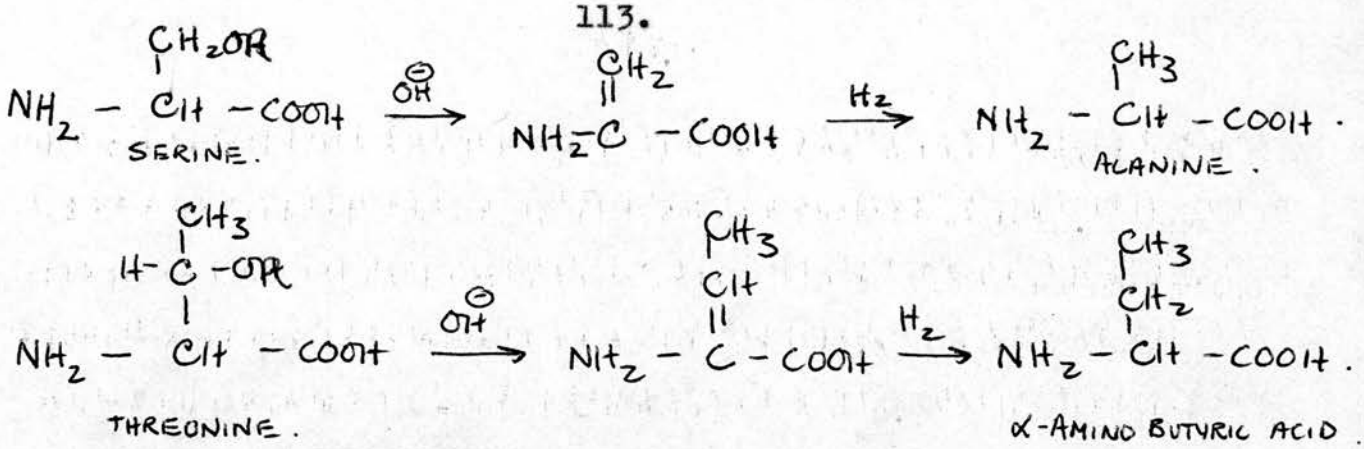
greater extent of cleavage and degradation into peptides of a much lower average molecular weight. Fractionation of the peptides was obtained by chromatography and electrophoresis. The spectral characteristics of the products of proteolysis after chromatographic separation were similar to the patterns after tryptic hydrolysis. On investigation of the fractions it was discovered that they were all complex mixtures which would be difficult to resolve. None of the fractions were noticeably richer in carbohydrate than the original protein and investigation of the components of each fraction by the chromatographic-electrophoretic technique and selective sprays indicated that nearly all the peptides resolved contained bound carbohydrate. Such investigations of the products of pronase digestion of phycoerythrin must lead us to the conclusion, as did the results of tryptic digestion, that the carbohydrate in phycoerythrin is not located in a single prosthetic group but must be widely distributed throughout the molecule. Furthermore, since the carbohydrate is bound in peptides which by nature of their electrophoretic, chromatographic and spectral characteristics prove to be other than chromopeptides, most of this carbohydrate must be linked to amino acid and not to the chromophore.

Certain linkages in glycopeptides are known to be alkali-labile and the series of experiments described and the methods of investigation of the products were designed to give some insight into the types of carbohydrate-amino-acid linkages present in phycoerythrin. Direct alkaline hydrolysis of

phycoerythrin with sodium hydroxide resulted in a fall in pH which must result from release of acid by the protein. One explanation of this is that the carbohydrate ester bound to the dicarboxylic amino acids must be released to give the free sugar and the free β- or γ-carboxyl groups. Investigation of the alkaline hydrolysate revealed only traces of free sugar although this was to be expected since the free sugars would be degraded to saccharinic acids under the conditions of hydrolysis and this would lead to further fall in pH.

Alkaline hydrolysis of a glycoprotein in the presence of a suitable reducing reagent would be expected to release sugar under the proposed scheme and these released sugars would be stabilized by reduction. In structural investigations in polysaccharide chemistry sodium borohydride has been used as such a reducing reagent. The possibility of alkaline hydrolysis of bonds other than carbohydrate-ester must be considered. Glycosidic ether bonds involving threonine and serine are alkali-labile and are liable to undergo β-alkoxy elimination as described by Anderson et al. Such elimination would not result in a fall in pH since no acid would be released on hydrolysis.

However, serine and threonine involvement with glycopeptide linkage may be established using the same technique, since the unsaturated products of β-elimination undergo stabilization similar to that of the sugar moiety.



β-alkoxy elimination accompanied by reduction results in the formation of alanine where serine was involved in a glycosidic linkage and in formation of α-amino butyric acid where threonine was involved.

Using this technique and conditions of reaction as developed by Pigman et al.[105] a series of experiments were carried out involving simultaneous alkaline hydrolysis and reduction. These resulted in the conclusions that threonine must take part in an ether bond but that serine does not. This was proved by the incorporation of tritium in the reaction products by sodium borotritilide.

The carbohydrate picture was not so clear and attempts to isolate the small amounts of carbohydrate from the highly ionic hydrolysates were unsuccessful. The fact that carbohydrate was released was ascertained by quantitative sugar estimation of the percentage carbohydrate in native phycoerythrin and in reduced phycoerythrin. This did indicate that alkaline hydrolysis had resulted in carbohydrate release. Whether this carbohydrate was released from a carbohydrate ester or from a glycosidic ether bond could not be determined.

The possibility also exists that the threonine is involved with linkage of the chromophore and not carbohydrate. Indeed, release of a green-fluorescing pigment could have resulted from alkaline elimination of a chromophore-threonine bond. Although it seems more likely, from the nature of the chromophore itself, that the threonine is bound to sugar, no rigorous conclusions can be made from the results available.

Digestion of the protein with the non-specific carbohydrase hemicellulase resulted in the hydrolysis of the protein-carbohydrate bonds. This indicated that the carbohydrate in phycoerythrin must be bound to the protein moiety by normal polysaccharide type linkages. The size of the residual protein moiety after the removal of carbohydrate and the low degree of proteolysis as a result of digestion indicated that the carbohydrate must be located at the exterior of the molecule. Attempts to isolate oligosaccharide material by a method involving a protein-carbohydrase digestion with dialysis was to some extent successful. Separation of this oligosaccharide from the monosaccharides also present in the hydrolysate/dialysate proved difficult and no additional conclusion to the results obtained from previous hydrolysates could be drawn. The oligosaccharide appeared to be an β-linked, glucose-containing disaccharide, probably cellobiose.

In the light of the results obtained, we can make the following conclusions regarding phycoerythrin:

1) 7% of the weight of the protein phycoerythrin is carbohydrate which is covalently bound to the protein moiety.

2) The nature of this carbohydrate is complicated and five common reducing sugars could be isolated from protein hydrolysates.

3) No amino sugars, acetylated amino sugars, or sulphated sugars could be detected.

4) It could be supposed from the complexity of the monosaccharide pattern that the degree of polymerisation of the sugar moiety must be low and isolation of no more complex oligosaccharide than a disaccharide from hydrolysates and enzyme digests seems to lend strength to this argument.

5) Analysis of the enzymic proteolysis products indicates that the carbohydrate is widely distributed throughout the molecule and is not located in only one or a few major prosthetic groups.

6) Most of the amino acid-carbohydrate linkages appear to involve the reducing ends of carbohydrate units since methylation of glycopeptides and subsequent methanolysis gave only fully methylated methyl glycosides. There is some possibility of bonding through two positions of arabinose.

7) Alkaline hydrolysis reveals that aspartic acid and glutamic acid or their amides seem to be involved in glycopeptide bonds. Serine is definitely not involved in other peptide than bonding; but threonine, if not bound to the

chromophore, is bound to carbohydrate through alkali-labile ether bonds.

8) In the light of the alkaline hydrolysis results reconsideration of the homogeneous glycopeptide isolated from the tryptic digest reveals that the linkage must involve glucose and aspartic acid either as a carbohydrate ester or as a glycosylaminyl bond to the amide of aspartic acid.

9) The carbohydrate is located at the periphery of the molecule and is accessible to the non-specific carbohydrase hemicellulase which further indicates that some of the carbohydrate is bound via expected polysaccharide type linkages.

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